Echocardiographic / Doppler criteria of normality, the findings in cardiac disease and the genetics of familial dilated cardiomyopathy in Newfoundland dogs

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Abstract

Dilated cardiomyopathy (DCM) is common in pedigree dog breeds including Newfoundlands. The breed predisposition and the familial prevalence within breeds support a genetic basis to the disease. Familial occurrence of DCM has only recently been recognised in man, and echocardiographic abnormalities are common in relatives of DCM patients.

Echocardiography is the method of choice for confirming the diagnosis of DCM. Echocardiographic/Doppler data are presented from 223 scans from 165 individual Newfoundland dogs. The scans were categorised into six groups based on the clinical presentation, M-mode echocardiographic results and the Doppler derived aortic velocity. The Normal group showed no abnormalities (n=86). The DCM (overt or occult) group had a rounded left ventricle and fractional shortening (FS) <22% (n=35). There were two depressed fractional shortening groups, without other abnormalities; one with FS less than 18% (dFS<18%) (n=29) and the other with FS 18-20% (dFS18-20%) (n=24). The left ventricular enlargement (LVE) group was defined as a LV diastolic dimension greater than 55mm (males) or >50 mm (females), without any M-mode evidence of systolic dysfunction (n=8). Dogs with an aortic velocity exceeding 1.7 m/s were defined as showing evidence of subaortic stenosis (SAS group) (n=40).

Data from complete echocardiographic/Doppler analysis of the Normal group were assessed for dependence on the gender, age and size of dog (weight or body surface area (BSA)) and the heart rate (mean R-R interval) by linear regression analyses. LV volumes and M-mode measurements were positively correlated with size. Gender was not an important predictor of most echo measurements once data was normalised for BSA. Advancing age was a significant negative predictor of LV volumes and dimensions although influence on wall thickness was not significant. Age also showed a significant influence on diastolic function, assessed by mitral inflow and pulmonary venous flow, similar to changes described in man.

The Newfoundland groups were compared. The DCM group had significantly increased LV volume and dimensions and decreased systolic function than other groups. There were few significant differences between the groups for diastolic function parameters. There was considerable overlap between groups for all dimensions and the parameters of systolic function, although the pre-ejection period: ejection time (PEP:ET) ratio appeared to be most sensitive for distinguishing normal from DCM dogs. In both dFS groups and the LVE group, this ratio was intermediate between the Normal and DCM groups, in contrast to other parameters of systolic function. Left atrial dysfunction was also identified in the DCM group, but was less marked in both dFS and the LVE groups. Some dogs in the LVE and dFS groups progressed to develop DCM but a longer duration of study would be required before firm conclusions can be drawn about progression.

Most of the dogs in this study were related. Pedigree and segregation analyses were supportive but not conclusive for an autosomal dominant mode of inheritance for DCM. A simulated linkage analysis indicated that this family was sufficiently informative for a genetic linkage analysis study. A pilot study assessing a number of anonymous canine microsatellites confirmed that there was sufficient heterozygosity and polymorphism, despite significant inbreeding, to permit a genetic linkage analysis study. No significant LOD score was achieved for the twelve microsatellites assessed. However, the available data indicate that a genome-wide linkage analysis is likely to be successful, with phenotyping based on the echocardiographic data.

Declaration

a) This thesis has been entirely composed by the author, Joanna Dukes McEwan.

b) The thesis details work that has been carried out in person by the author, Joanna Dukes McEwan. Where assistance, advise or participation of other individuals or institutions were sought, this is acknowledged both in the text and with the acknowledgements. The string phantom experiments were carried out with Dr. Carmel Moran and Mr. Tom Anderson of the Department of Medical Physics and Medical Engineering, The Royal Infirmary, University of Edinburgh. The repeatability study was carried out in collaboration with Ms. Anne French of the Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies, University of Edinburgh. Dr. Peter Teague from the Medical Research Council Human Genetics Unit, Western General Hospital, Edinburgh, carried out the initial simulated linkage analysis.

Signed..... .....

Joanna Dukes McEwan

Date.....27/4/99.....

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Further training in the computer software and the principles of linkage analysis was provided during two courses I attended, provided by the Human Genome Mapping Project Resource Centre, Hinxton, Cambridgeshire.

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DEDICATION

Brian and Pat Dukes

This thesis is dedicated to my parents for their support and encouragement over many years.

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LIST OF ABBREVIATIONS USED IN TEXT AND TABLES

Abbreviation	Full name
acc. or accel	Acceleration time
ACE	Angiotensin converting enzyme
ACEI	Angiotensin converting enzyme inhibitor
af or AF	atrial fibrillation
ANOVA	Analysis of variance (one way)
Ao	Aorta / Aortic
Aod	Aortic diameter in diastole
AoI or AoR	Aortic insufficiency or aortic regurgitation
Aov	Aortic peak velocity
Aovti	Aortic flow velocity time integral
Arv	Pulmonary venous flow Ar wave velocity
ARVD	Arrhythmogenic right ventricular cardiomyopathy (dysplasia)
Av	Mitral (or tricuspid) A wave velocity
Avti	Mitral A wave velocity time integral
bpm	Beats per minute (heart rate)
BSA	Body surface area
CFDE	Colour flow Doppler echocardiography
CHF	Congestive heart failure
c.o.v.	Coefficient of variation (Repeatability study; Serial scans)
cPEP	Corrected pre-ejection period
CRF	Chronic renal failure
CW	Continuous wave Doppler
d	Diastole
2D	Two dimensional echocardiography
DCM	Dilated cardiomyopathy
decel	Deceleration time
dFS	Depressed fractional shortening
1degAVB	1st degree atrioventricular block
%diffMeans	Percentage difference between Means (Serial scans)
DJD	Degenerative joint disease
dP/dt	Rate of rise of pressure in LV
dur	Duration
Dv	Pulmonary venous flow peak D wave velocity
Dvti	PVF D wave velocity time integral
dv/dt	Acceleration of aortic flow (peak = maximum (max), or mean (mn))
EDV	End-diastolic volume
EDVI	End diastolic volume index (volume /m^2)
EF	Ejection fraction
EFteich	Ejection fraction calculated by the Teicholz method
EPSS	Mitral valve M-mode E point to septal separation
ESV	End-systolic volume
ESVI	End systolic volume index (volume /m^2)
ET	Ejection time
Ev	Mitral (or tricuspid) E wave peak velocity
Evti	Mitral (or tricuspid) E wave velocity time integral
excl.	Excluded
FDCM	Familial dilated cardiomyopathy
FS%	Fractional shortening
GA	General anaesthesia
GDV	Gastric dilatation / volvulus
HCM	Hypertrophic cardiomyopathy

HR	Heart rate
id.	Identity
ILP	Idiopathic laryngeal paralysis
incl	Included
IVRT	Isovolumic relaxation time
IVSd/s	Interventricular septum thickness diastole / systole
LA	Left atrium
LAad/s	Left atrial area in diastole / systole
LAd	Left atrial dimension, diastole (short axis 2D view)
LAs	Left atrial dimension, systole (M-mode measurement)
LAEI	Left atrial emptying index = (systolic area - diastolic area)/systolic area
LAld/s	Left atrial length (width) above and parallel to mitral annulus in diastole / systole
L.Ap.	Left apical view
lat	Lateral
LGMD	Limb-girdle muscular dystrophy
LOD	Logarithm of the odds
LPS	Left parasternal view
LV	Left ventricle
LVad/s	Left ventricular area in diastole / systole
LVdv	Left ventricular diastolic volume
LVE	Left ventricular enlargement
LVFW	Left ventricular free wall (=left ventricular posterior wall)
LVIDd/s	Left ventricular internal dimension in diastole / systole
LVld/s	Left ventricular length in diastole / systole
LVpwd/s	Left ventricular posterior wall (=left ventricular free wall) in diastole / systole
LVsv	Left ventricular systolic volume
M or M-	M-mode parameter
MAM	Mitral annulus motion
MD	Muscular dystrophy
ME or MA	Mitral E wave or A wave parameter
MR	Mitral regurgitation
m	Metres
m/s	Metres per second
MV	Mitral valve
NAD	No abnormalities detected
nr	Not recorded / not reported
ns	Not significant
NYHA	New York Heart Association (classification of congestive heart failure)
occ.	Occasional
PA	Pulmonary artery
PAv	Pulmonary artery peak velocity
PCWP	Pulmonary capillary wedge pressure
PEP	Pre-ejection period
PEP:ET	Pre-ejection period : ejection time ratio
periph.neuro.	Peripheral neuropathy
PI	Pulmonic insufficiency
PLCVC	Persistent left cranial vena cava
PM	Post-mortem examination
Prev.	Previously
PTS	Put to sleep (euthanased)
PVF	Pulmonary venous flow
PM	Post mortem examination
PW	Pulsed wave Doppler echocardiography
RAE	Right atrial enlargement
Resp	Respiratory

RPS	Right parasternal view
R-R	Mean interval between R waves on ECG
RVd	Right ventricular dimension, diastole
RVE	Right ventricular enlargement
s	Systole
s	Second(s)
SAS	Subaortic stenosis
S/C	Subcostal view
sd	Standard deviation
sd%Mean	Standard deviation expressed as a percentage of the Mean
sep	septal
sh.ax.	Short axis view
sig	Significant
Simp	Simpson's rule
sq.	Squared
sq.rt. or √	Square root
STI	Systolic time intervals
SVPC(s)	Supraventricular premature complex(es)
Sv	Pulmonary venous flow peak S wave velocity
Svti	PVF S wave velocity time integral
SV	Stroke volume (of left ventricle)
SVI	Stroke volume index (volume / m^2)
T or TV	Tricuspid valve
Teich	Teicholz method of measuring ejection fraction
TR	Tricuspid regurgitation
%thIVS	Percentage thickening of interventricular septum
%thLVpw	Percentage thickening of left ventricular posterior wall
v	Velocity
Vcf	Velocity of circumferential fibre shortening
vol	Volume
VPC(s)	Ventricular premature complex(es)
Vtach	Ventricular tachycardia
vti	Velocity time integral
Wt	Weight

A Newfoundland dog

Aims of this study

(i) A major aim of this study was to produce echocardiographic / Doppler criteria of normality for the specific breeds susceptible to dilated cardiomyopathy and to identify factors affecting the various parameters.

(ii) To examine the use of echocardiography in screening dogs for evidence of occult dilated cardiomyopathy in breeds susceptible to dilated cardiomyopathy.

(iii) To carry out serial evaluation of individuals with echocardiographic parameters which were abnormal to determine the significance of such abnormalities and whether they allow prediction of occult dilated cardiomyopathy.

(iv) To determine the mode of inheritance of familial dilated cardiomyopathy in susceptible breeds and to ascertain whether the family under investigation would be suitable for a genetic linkage analysis study.

NOTE: The study presented in this thesis concentrated on the above aims for the Newfoundland breed.

SECTION A

INTRODUCTION

IDIOPATHIC DILATED CARDIOMYOPATHY: GENERAL REVIEW

A.1. *Definition*

Cardiomyopathies are defined as diseases of the myocardium associated with cardiac dysfunction (Richardson *et al* 1996). The original World Health Organisation classification included three major morphological types of cardiomyopathy; dilated, hypertrophic and restrictive. The new classification is by dominant pathophysiology or, where possible, by aetiological / pathogenetic factors (Richardson *et al* 1996). The 1995 World Health Organisation / International Society and Federation of Cardiology Task Force on the definition and classification of cardiomyopathies defined dilated cardiomyopathy as being *characterised by dilatation and impaired contraction of the left ventricle or both ventricles* (Richardson *et al* 1996). A number of different definitions of dilated cardiomyopathy are present in the literature, but it is striking that rather than an actual definition, most authors, as in the WHO classification, describe the condition as being characterised by left ventricular dilatation and dysfunction (Thomas 1987; Calvert 1995b; Monnet *et al* 1995; Sisson & Thomas 1995; Freeman *et al* 1996). One of the best working definitions of the canine disease was given by Cobb (1992) who defined idiopathic dilated cardiomyopathy as a "condition of unknown aetiology characterised by a progressive dilatation of one or both ventricles with severe impairment of systolic function and a high end-diastolic pressure, excluding congenital, coronary arterial, hypertensive, vascular, pulmonary parenchymal, valvular or other cardiovascular disorders". This was based on the definition by Wynne and Braunwald (1992). Some authors include the presence of arrhythmias and the poor prognosis in the definition (Freeman *et al* 1996), some note that left atrial dilatation is usually present (Ware & Bonagura 1986) and others include in the definition the presence of some myocardial hypertrophy (Keene 1989; ISACHC 1995).

Idiopathic dilated cardiomyopathy is probably not a single disease but the result of a variety of different pathological processes or defects in myocardial metabolism leading to chronic end-stage organ failure (Keene 1989). Primary idiopathic dilated cardiomyopathy is a diagnosis of exclusion (Thomas 1987) and greater understanding of this condition will result in expansion of some of the secondary cardiomyopathy categories.

Secondary cardiomyopathies may be categorised by aetiology and have been listed (Church 1980; Wood 1983; Ware & Bonagura 1986; Thomas 1987; Fox 1988; Fox 1989; Cobb 1992; Calvert 1995a; Sisson & Thomas 1995). Secondary cardiomyopathies will expand with increased understanding of the aetiopathogeneisis of idiopathic dilated cardiomyopathy.

A.2. *Canine idiopathic dilated cardiomyopathy*

A.2.1. *A historical perspective*

Idiopathic dilated cardiomyopathy (DCM) was first reported in the veterinary literature in 1970 (Ettinger *et al* 1970; Ettinger & Suter 1970) having been recognised in the US in the late 1960s (Thomas 1987). Atrial fibrillation was said to be a constant feature of DCM in the earliest literature (Ettinger & Suter 1970). Initial veterinary reports stated that right sided heart failure signs preceding left sided failure (Ettinger *et al* 1970; Ettinger & Suter 1970). This discrepancy with what is generally appreciated about the disease today, with a predominance of left sided heart failure (Sisson & Thomas 1995) may reflect improved clinical recognition of the disease and earlier diagnosis.

Even shortly prior to 1970, the veterinary literature did not recognise any syndromes typical of DCM (Detweiler & Patterson 1965; Luginbühl & Detweiler 1965; Bishop 1971). Atrial fibrillation was treated as a "primary diagnosis" in the early literature. Ettinger (1971) described atrial fibrillation occurring secondary to left ventricular dilatation in middle aged giant breed dogs but did not specify any underlying myocardial disease or cardiomyopathy. In a series of cases with atrial fibrillation

cases reported by Bohn and colleagues (1971), a high breed specific prevalence rate of atrial fibrillation in giant breeds such as Newfoundlands, great Danes, St. Bernards, Irish Wolfhounds and also the Dobermann was recorded, and it is reasonable to speculate with the benefit of hindsight that many of these dogs had DCM. The poor prognosis associated with congestive failure and atrial fibrillation was noted by Bohn and colleagues (1971) although these authors also comment on the improved prognosis associated with atrial fibrillation in giant breeds of dogs. A trend to increased survival in dogs with atrial fibrillation was noted to be weakly correlated to body weight by Boevé and others (1984).

In the early literature, there appears to have been some confusion about the diagnosis of DCM and distinguishing it from congenital heart disease, especially conditions resulting in mitral insufficiency (Hamlin *et al* 1965; Hamlin & Harris; 1969; Dear 1971; Lord 1974).

Other early case reports detailing the findings in DCM are sparse. Church *et al* (1976) published a case report in an Irish Setter and Wilkes (1980) detailed the findings in giant breeds of dogs. The condition is covered by book chapters (Dear 1974; Ettinger 1975; Ogburn 1977; Bond & Tilley 1980; Harpster 1983; Wood 1983; Tilley *et al* 1983) and Lord (1974; 1976) published results of some impressive observations about assessment of systolic and diastolic function in DCM.

The literature variously refers to the condition as cardiomyopathy (Darke & Else 1984) (unclassified), idiopathic cardiomyopathy (Ettinger & Suter 1970; Ettinger *et al* 1970), myocardiopathy (Dear 1971; Otter 1991); congestive cardiomyopathy (Ogburn 1977; van Fleet *et al* 1981), idiopathic congestive cardiomyopathy (Ettinger 1975; Church *et al* 1976; Wilkes 1980; McCarthy 1984; Thomas 1987b), congestive (dilated) cardiomyopathy (Wood 1983), dilated cardiomyopathy (Lombard 1984a) and idiopathic dilated cardiomyopathy (Cobb 1992). The latter term is now generally accepted in the human and veterinary literature, although with increasing knowledge

about aetiological factors, fewer cases are likely to be defined as idiopathic in the future.

A.2.2. *Prevalence of canine dilated cardiomyopathy*

Few estimates of the incidence or prevalence of DCM have been published although Keene (1989) felt that the incidence was increasing. Sisson & Thomas (1995) published details about the prevalence of this condition in the United States, deriving information from the Veterinary Medical Database at Purdue University for 1986 - 1991.

The overall prevalence was about 0.5% although if this was analysed further, prevalence rates were much higher in pedigree dogs (0.65%) than mixed breed dogs (0.16%). They confirmed that it was predominantly a disease of large and giant breeds and dogs under 12 kg were rarely affected. Overall, males were predisposed (0.66%) compared with bitches (0.34%) although it was noted that where the inclusion was dependent on identification of the presence of congestive heart failure, this may underestimate the prevalence in females (Sisson & Thomas 1995).

Breed specific prevalences were recorded. DCM appeared to be most prevalent in Deerhounds (6.0%) although numbers were low compared with the other predisposed breeds. In order of frequency, breed prevalence was 5.8% Dobermanns, 5.6% Irish wolfhounds, 3.9% great Danes, 3.4% boxers, 2.6% St. Bernards, 1.7% Afghan hounds, 1.3% Newfoundlands, 0.9% old English sheepdogs, 0.69% English cocker spaniels and 0.34% American cocker spaniels. The prevalence increased with age. Boxers, Dobermanns and great Danes were analysed in different age groups, which showed a trend to increasing prevalence in older dogs.

A.2.3. *Prevalence in Man*

In man, a prevalence of DCM has been quoted as 36.5/100 000 with an annual incidence of 8 per 100 000 in the US literature (Wynne & Braunwald 1997). In Sweden, an autopsy survey gave an incidence of 5 per 100 000. These figures are felt

to be an underestimate because DCM has a long preclinical phase during which the patient is asymptomatic and remains undiagnosed (Keeling & McKenna 1994). In an Italian study, with relative screening of new cases, the autopsy incidence (4.5 / 100 000 / year) and the new cases diagnosed (2.45 / 100 000 / year) were added to give an annual incidence rate of 6.95 cases per 100 000 population per year (Rakar *et al* 1997), although this addition was criticised as potentially over-estimating cases by Tavazzi (1997). There is increased risk of disease in males and the black population (Mestroni *et al* 1994b). DCM is the leading reason for cardiac transplantation in the world (Mestroni *et al* 1994b).

A.3. *Aetiology of DCM*

By definition, idiopathic DCM is of unknown aetiology, although this may merely reflect the current lack of understanding of aetiopathogenesis (Cobb 1992). Indeed, with increasing understanding of the aetiology and pathogenesis, the difference between cardiomyopathy and specific heart muscle disease has become indistinct (Richardson *et al* 1996). It is probable that the condition does not reflect a single clinical entity but represents end-stage myocardial failure resulting from a wide variety of disorders (Thomas 1987; Keene 1989). Indeed, the original myocardial insult may not have been recognised and the offending cause is no longer apparent at the time of presentation (Cobb 1992) and end-stage human DCM often cannot be linked to a specific aetiology (McMinn & Ross 1995). Some potential aetiological factors have been reviewed (Cobb 1992). One or more subcellular metabolic abnormalities are suspected as being responsible for the myocardial failure (Thomas 1987; Sisson & Thomas 1995; ISACHC 1995). Dear (1974) showed foresight in describing that the idiopathic cardiomyopathies probably had an underlying metabolic (biochemical) or an ultrastructual defect. The fact that the disease occurred predominantly in large, fast growing breeds of dog led Bond and Tilley (1980) to postulate hereditary and nutritional factors. An inherited or genetic basis to canine DCM has received increasing support, as reviewed in Section C of this thesis.

In man, many factors, alone or in combination, can contribute to the development of DCM, including excessive alcohol consumption (McKenna *et al* 1998), hypertension, myocarditis, pregnancy, diabetes mellitus, doxorubicin therapy and secondary to a generalised disorder (Abelmann & Lorrell 1989; McMinn & Ross 1995).

Currently, three major aetiopathogenetic mechanisms are hypothesised in human DCM (Mestroni *et al* 1994a;b):

(i) Enteroviral infection with viral persistence leading to chronic myocardial damage

(ii) Autoimmune heart disease, with humoral and cellular immune dysfunction

(iii) The presence of a primary genetic defect which could act separately or in association with one of the categories above.

These three pathogenetic factors may all be closely linked (Keeling & McKenna 1994). Detailed discussion of the possible aetiology is beyond the scope of this thesis. The evidence for a genetic basis to DCM is reviewed and discussed in Section C of this thesis.

A.4. *Biochemical defects associated with canine DCM*

Biochemical abnormalities have been reported in association with DCM (Bishop 1987). More specific biochemical abnormalities have been found in some dogs with DCM which include a variety of abnormalities in Dobermann DCM. Although biochemical abnormalities may offer evidence for a genetic basis for DCM, they may merely be a consequence of heart failure.

A.4.1. *Biochemical defects in energy production within the myocardium of cardiomyopathic Dobermanns*

McCutcheon and colleagues (1992) investigated the hypothesis that a myocardial metabolic defect was associated with naturally occurring Dobermann DCM. In DCM, the myocardium had greatly reduced mitochondrial electron transport activity (50% reduced) and myoglobin concentration (90% reduced). The myoglobin deficiency was investigated in further detail. O'Grady and others (1992) reported that myocardial myoglobin deficiency may be an aetiological factor for Dobermann DCM and that normal Dobermanns had lower myoglobin concentration than mongrels, associated with subclinical systolic dysfunction in this breed. Myoglobin levels decrease in all models of heart failure, and were correlated with mitochondrial and sarcoplasmic reticular ATPase activities and parameters of cardiac performance (O'Brien *et al* 1992). Myoglobin is known to protect the heart from hypoxia by functioning as a sarcoplasmic oxygen reservoir and shuttle. Myoglobin deficiency was implicated in the pathogenesis of congestive heart failure and possibly the predisposition of Dobermanns to DCM (O'Brien *et al* 1992).

DCM is known to be associated with derangement of myocardial sarcoplasmic calcium (Ca) homeostasis and energy production. O'Brien and co-workers (1995) investigated calcium cycling of the sarcoplasmic reticulum, by assessing both mRNA and expression of the Ca release channel and Ca ATPase of the sarcoplasmic reticulum. An asymmetric down-regulation of the Ca cycle was identified, with the Ca channel more depressed than the Ca ATPase. The mRNA and enzyme contents of the markers of the glycolytic and oxidative phosphorylation pathways were down-regulated in DCM, but the markers of the TCA (tricarboxylic acid) cycle and ribosomal RNA were up-regulated. Another interesting observation was that mRNA was more degraded in cardiomyopathic than normal hearts. These authors concluded that transcriptional and translational responses to the pathophysiology of cardiomyopathy are responsible for the major determinants of the metabolic responses seen in heart failure. Changes specific to the entity of Dobermann DCM were not recognised.

The cardiomyoctye cellular energy deficiency associated with Dobermann DCM, evidenced by these various biochemical abnormalities, was postulated to be due to an abnormality of the creatinine kinase (CK) system, which plays a dominant role in energy cycling (O'Brien 1997). In this system, the mitochondrial isoenzyme of CK uses ATP to produce creatine phosphate, which is utilised by the myofibrillar isoenzyme of CK (CK-MM) and the cytosolic isoenzyme of CK (CK-MB) to replenish ATP that has been hydrolysed during contractile and ion-transport activities. In Dobermann DCM, cytosolic CK-MB was significantly reduced, by 50% compared with controls. This relative deficiency of CK-MB may have a central role in the energy deficiency associated with this condition. It is in marked contrast to the relative increase in CK-MB associated with a decrease in total CK in human and Syrian hamster DCM but is similar to the findings of cardiomyopathy associated with diabetes mellitus or dietary restriction. O'Brien (1997) therefore concluded that these changes may be related to deficiency of an energy substrate or of delivery of this substrate within the cardiomyocyte.

Although biochemical defects as a result of a genetic abnormality are an attractive hypothesis supporting the genetic aetiology of DCM, these results demonstrate that caution must be urged in differentiating identified biochemical abnormalities as a *consequence* of the heart disease rather than the *cause* of the heart disease.

Some abnormalities in energy production have also been implicated in human DCM. Bonnet and colleagues (1998) metabolically screened consecutive childhood cardiomyopathies. They assessed mitochondrial respiratory chain complex activity in endomyocardial or skeletal muscle biopsies, and found respiratory chain enzyme deficiency in seven patients, with defects confined to the myocardium in six of these children. Antozzi and Zeviani (1997) reviewed the disorders of cardiac energy metabolism, including defects of mitochondrial oxidative phosphorylation.

A.4.2. *Altered β receptor function*

Hoey and colleagues (1991) reported on the findings from four dogs with DCM. β_1 receptor density was preserved in the left ventricle although β_2 density was reduced. A reduction in cyclic AMP production stimulated via adenylate cyclase was interpreted to reflect a post receptor defect or dysfunction of a catalytic subunit of adenylate cyclase.

Tarducci and colleagues (1998) compared the β adrenergic receptor density on lymphocytes in six great Danes with cardiomyopathy and six normal great Danes. A significant reduction in density of both total β receptors, and β_1 and β_2 receptor subtypes was identified. This is consistent with prolonged sympathetic stimulation or to the presence of auto-antibodies against the β receptor.

A.4.3. *Myocardial L-carnitine deficiency*

Myocardial L-carnitine deficiency has been identified in a family of Boxers with DCM (Keene *et al* 1991). L-carnitine (3 hydroxy 4-N-trimethylaminobutyric acid) is synthesised in the liver and is soluble in the plasma, concentrating in skeletal and cardiac myocytes by an ill-defined transport mechanism. Adequate myocardial concentration of L-carnitine has been shown to be critical for fatty acid metabolism. Long chain free fatty acids are quantitatively the most important energy producing substrate in the myocardium (Keene 1991). Free fatty acids are esterified and L-carnitine is required to catalyse their transfer across the outer and inner mitochondrial membranes, to release acetyl CoA for β oxidation of fats and energy production. Normally, plasma and myocardial L-carnitine levels are closely correlated (Pion *et al* 1998). As plasma levels of L-carnitine are usually normal or even high in affected dogs while endomyocardial biopsy confirmed low myocardial L-carnitine levels, it is thought that an abnormality of the membrane transport from plasma into myocardial cells underlies this form of myocardial failure (Keene 1991; 1992), which is certainly familial and possibly inherited in the Boxer breed (Keene *et al* 1991). Supplementation of L-carnitine at 220 mg/kg per day orally divided as three doses results in clinical improvement after one to four weeks and echocardiographic

improvement from two to three months of the start of dosing, although after six months of treatment, they plateau with generally subnormal left ventricular function. Some Dobermanns may have myocardial L-carnitine deficiency (Keene *et al* 1989).

In man, L-carnitine deficiency has been associated with familial DCM in children, where an abnormality of renal tubular reabsorption of L-carnitine and increased fractional excretion of carnitine was believed to be responsible for one case (Waber *et al* 1982). Elimination of carnitine deficiency as a factor in familial DCM in man is considered mandatory (McMinn & Ross 1995; Bonnet *et al* 1998). As well as DCM, other systemic signs reported in some affected children include hypoketotic hypoglyaemia and a skeletal myopathy (Antozzi & Zeviani 1997).

There is no evidence that carnitine supplementation in the absence of myocardial deficiency has any beneficial effect (Keene 1992). L-carnitine is expensive to use routinely in cases where documented deficiency has not been shown (Keene 1991). However, Abelmann and Lorrell (1989) reviewed the literature suggesting that supplementation in experimental doxorubicin-induced cardiomyopathy or Syrian hamster cardiomyopathy may be beneficial.

A.4.4. *Taurine deficiency*

Since a reversible dilated cardiomyopathy associated with dietary taurine deficiency and supplementation was reported in cats (Pion *et al* 1987), this condition is now of greatly reduced incidence in this species following action by pet food manufacturers (Pion 1998). Although the high concentration of taurine in the myocardium is appreciated, the role played by taurine is poorly understood (Pion *et al* 1998). It is believed to play a role in inotropy, antiarrhythmia, enhanced sarcolemmal calcium binding properties, carbohydrate metabolism and osmotic regulation (Kramer *et al* 1995). Human patients with heart failure and some dogs with valvular heart disease are reported to have increased myocardial taurine concentrations (Pion *et al* 1987; Kramer & Fox 1989; Kramer *et al* 1995) and this is believed to be a compensatory mechanism. Most dogs with DCM do not have decreased plasma levels of taurine;

indeed levels may be significantly increased (Kramer & Fox 1989), but American cocker spaniels and golden retrievers with DCM may have a low plasma taurine (Kramer *et al* 1995). The association between low plasma taurine concentration and American cocker spaniel DCM has been proposed although this breed did not appear to respond to taurine supplementation as convincingly as cats (Pion *et al* 1998). However, when taurine deficient (<50 nmol/ml) American cocker spaniels were treated with both taurine and L-carnitine in a double blind, placebo controlled study, echocardiographic and clinical improvement was recognised (Kittleson *et al* 1997).

There has been some controversy over whether taurine levels should be assessed in whole blood or plasma. Sanderson and colleagues (1998) suggested that both should be done simultaneously to accurately predict tissue (cardiac or skeletal muscle) levels. Heparinised samples are normally obtained (Pion 1998).

A.4.5. *Other nutritional factors*

Nutritional factors were also implicated in a report on DCM in nine Dalmations which were fed a prescription diet to treat or prevent urate urolithaisis, common in the breed as a familial abnormality of urate metabolism (Freeman *et al* 1996). This diet is restricted in proteins, nucleic acids, calcium, phosphate, magnesium, sodium and copper. However, it appears that the probable aetiology of the cardiomyopathy in these dogs is due to urinary loss of other amino-acids including taurine and carnitine (Pion *et al* 1998) and these authors urged that breeds of dog with the potential for developing urate or cysteine urolithiasis should receive taurine and carnitine supplementation if DCM is acquired, as well as conventional medication.

Selenium and thiamine deficiencies are known to cause or contribute to the development of DCM in various species (Abelmann & Lorrell 1989; Freeman *et al* 1996).

A.5. *Pathophysiology of dilated cardiomyopathy*

Various aspects of the pathophysiology of DCM are discussed in the literature (Bonagura & Ware 1986; Thomas 1987; Fox 1988; Sisson & Thomas 1995; Calvert 1995b; ISACHC 1995). DCM is associated primarily with systolic or pump failure which initially and predominantly affects the left ventricular myocardium. Impaired contractility results in decreased stroke volume and decreased rate of rise of left ventricular pressure (dP/dt). Increased left ventricular end-systolic and end-diastolic volumes result (volume overloading), causing increased wall stress with compensatory sarcomere replication in series (eccentric hypertrophy) (La Place relationship). Reduced stroke volume mediates a baroreceptor response, reflexly increasing sympathetic drive (and withdrawal of vagal tone). β_1 stimulation results in increased heart rate (and contractility where there is sufficient myocardial reserve) and stimulation of renin release from the juxtaglomerular apparatus of the kidney. α_1 stimulation results in arteriolar constriction, increasing peripheral vascular resistance to maintain blood pressure and venoconstriction, increasing preload thereby attempting to improve stroke volume by the Frank-Starling mechanism. Renin release is also stimulated by reduced renal perfusion pressure, and these factors activate the renin-angiotensin-aldosterone system (RAAS). Angiotensin II has a number of potent effects. It is a potent vasoconstrictor, augmenting the α adrenergic effects. It increases synthesis and release of aldosterone resulting in increased sodium and water retention, which increases preload. Angiotensin II also has effects on the central nervous system causing increased thirst, increased synthesis and release of vasopressin (antidiuretic hormone) and increasing sympathetic drive further. Angiotensin II also plays a role in myocardial and vascular remodelling. The cardiovascular system is able to compensate for the disease initially and dogs remain asymptomatic for a considerable time as a result of various neurohormonal compensatory mechanisms. Eventually the effects become detrimental in the chronic setting and the patient shows overt signs of congestive heart failure.

Left ventricular dilatation eventually is out of proportion to hypertrophy and as left ventricular end-diastolic pressure increases, filling pressures increase, resulting in

increased left atrial pressure and gradual dilatation. From La Place's relationship, it can be appreciated that the left ventricular dilatation with relatively thin walls increases wall stress. The atrioventricular annulus becomes dilated and this, possibly in association with papillary muscle dysfunction, results in mitral regurgitation, exacerbating left atrial / left ventricular volume overload. Pulmonary venous congestion and pulmonary oedema result from left sided heart failure. Increased afterload and wall stress causes increased myocardial oxygen consumption and areas of ischaemia or overt myocardial damage may result in ventricular ectopy. The incessant tachycardia recognised in patients with high sympathetic drive due to congestive heart failure also increases myocardial oxygen consumption as well as compromising diastolic function. Increased left atrial size and pressure may cause the development of atrial fibrillation especially in giant breeds. Atrial fibrillation is a haemodynamically important arrhythmia in dilated cardiomyopathy as it may reduce the cardiac output by up to 25% by the mechanisms of:

(i) loss of atrial contraction and atrioventricular synchronisation
(ii) increased ventricular rate with reduced diastolic filling time
(iii) the variable R-R interval resulting in erratic cardiac filling (Bonagura & Ware 1986)
(iv) In addition, the persistent tachycardia usually recognised with atrial fibrillation may cause or exacerbate signs of congestive heart failure, as in paced canine models of congestive heart failure (Allworth *et al* 1995).

A.6. *Clinical signs associated with DCM*

The presenting signs are well described in text books and include weakness, lethargy, dyspnoea, exercise intolerance, cough, anorexia, weight loss, abdominal distension and syncope (Bond & Tilley 1980; Wilkes 1980; Darke & Else 1984; Darke 1985; Ware & Bonagura 1986; Thomas 1987; Fox 1988;1989; Keene 1989; Calvert 1995b; Sisson & Thomas 1995). Weight loss and skeletal muscle wasting is often dramatic (Keene 1989). Most clinicians consider this finding to be associated with a grave

prognosis although weight loss was not statistically found to be an prognostic indicator of survival (Monnet *et al* 1995).

Clinical examination of the dog with congestive heart failure due to DCM is usually characteristic for the condition (Darke & Else 1984; Bond, 1985; Darke 1985; Ware & Bonagura 1986; Thomas 1987; Fox 1988;1989; Calvert 1995b; Sisson & Thomas 1995). Clinical findings include the following: pale mucous membranes, sluggish capillary refill, weak peripheral pulse and cold extremities, weak precordial impulse, quiet or muffled heart sounds, irregular heart rate and pulse rate, variable pulse volume and pulse deficit, chaotic rhythm or ectopy, soft systolic murmurs and diastolic gallop sounds. Clinical signs of left sided congestive failure include a shallow tachypnoea to overt dyspnoea with ausculatory evidence of pulmonary oedema. Clinical signs of right sided heart failure include jugular venous distension, hepatomegaly, a positive hepatojugular reflux and abdominal distension due to ascites. A pleural effusion may be appreciated. Sometimes the clinical signs due to low cardiac output predominate over the congestive signs (cardiogenic shock). Muscle wasting may be clinically evident, especially over the temporal muscles and the thoracic and lumbar spinal epaxial muscles.

The presence of cardiac disease, evidence of cardiomegaly and the severity of clinical signs are variously used to classify heart failure in the New York Heart Association (NYHA) (Keene & Bonagura 1995) or the newer International Small Animal Cardiac Health Council (ISACHC) classifications (ISACHC 1995).

A.7. *Diagnosis of DCM*

Lombard (1984a) wrote that the clinical diagnosis of DCM may be based on signalment (particularly breed), the presence of congestive failure (left sided or generalised), radiographic evidence of moderate to severe cardiomegaly and the absence of loud heart murmurs suggesting congenital heart disease or acquired valvular heart disease. Even today, with more sophisticated techniques available, it is still a diagnosis of exclusion. However, standard diagnostic tests used include

electrocardiography, radiography and echocardiography and overall patient health and consequences of heart failure or drug treatment are assessed by clinical pathology. In man, the diagnosis of DCM is based on physical and non-invasive (echocardiographic) examinations with investigations to exclude other disease (Mestroni *et al* 1994b). There are practical difficulties in making the diagnosis of DCM, as recognised in humans by Keeling and McKenna (1994). In man, the exclusion of coronary artery disease and hypertensive disease is required (both uncommon in the dog) as well as valvular and pericardial disorders and other specific heart diseases including myocarditis and alcohol induced cardiomyopathy. In man, selective coronary artery angiography, indicated to exclude coronary artery disease, is required to confirm a diagnosis of DCM and in many centres, so is endomyocardial biopsy to exclude myocarditis (Keeling *et al* 1995), although this is still controversial (Keeling & McKenna 1994). There have been occasional reports of myocardial infarction in dogs (e.g. DeFrancesco *et al* 1996). Myocarditis appears to be more rare in dogs than in man. The technique of obtaining endomyocardial biopsy samples from dogs has been described (Keene *et al* 1990) and is not without risk. The importance of strict diagnostic criteria for DCM has been emphasised (Keeling & McKenna 1994; Keeling *et al* 1995).

In dogs, the signalment, history and clinical signs will usually result in a high index of suspicion about the diagnosis of DCM. Most cardiologists will perform ancillary examinations to confirm the diagnosis and to guide therapeutic options. These techniques have been reviewed in an number of texts (Thomas 1987; Fox 1988; Fox 1989; Calvert 1995b; Sisson & Thomas 1995). They include electrocardiography, signal averaged electrocardiography (Calvert 1992; 1995a;b; Calvert *et al* 1998a;b; Jacobs & Calvert 1998), Holter monitoring (Calvert 1992; 1995a;b; Moise & Defrancesco 1995; Goodwin 1998), radiography and echocardiography. These techniques will not be reviewed in detail in this thesis, with the exception of echocardiography (see Section B of this thesis). Echocardiography is considered to be the gold standard in the diagnosis of DCM (ISACHC 1995).

A.8. *Pathology of DCM*

In their series of fifty cases of presumed DCM in dogs, Darke and Else (1984) reported on the post mortem findings from eleven cases. Marked cardiac enlargement was reported associated with a flaccid heart. Left ventricular dilatation was sometimes associated with concurrent right ventricular dilatation giving a bifid apex. The ventricular walls are relatively thin although myocardial hypertrophy is present with increased heart weight: body weight ratio (Thomas 1987). Papillary muscles and trabeculae in the ventricles can appear flattened and atrophic (Bond & Tilley 1980; Sisson & Thomas 1995). The atrioventricular annulus is often proportionately more dilated than the chambers (Fox 1988; Sisson & Thomas 1995).

In human DCM, histopathological changes are reported to be common but non-specific. The 1995 WHO definition of DCM included non-specific histological findings as a criterion of diagnosis (Richardson *et al* 1996). They include myocyte hypertrophy, nuclear changes, myofibrillar loss, mitochondrial changes and variable degrees of fibrosis (Mestroni *et al* 1994b). The myocyte hypertrophy is determined by the increased nuclear DNA content, although myocyte width may be normal or even reduced (attenuation). It is uncertain whether this is a consequence of increased cell length or cell slippage (Davies & McKenna 1994) although a recent abstract suggested that increased length was responsible (Kaprielian *et al* 1998). Myocyte myofibrillary loss is reported as well as varying degrees of interstitial fibrosis (Davies & McKenna 1994). In the human disease, increased numbers of T lymphocytes are reported, although this finding varies considerably from case to case (Davies & McKenna 1994).

In dogs, it is remarkable how much relatively normal myocardium is present in a patient dying of myocardial failure (ISACHC 1995). Myocardial cells may be longer than normal, characteristic of eccentric hypertrophy (ISACHC 1995). Histopathological changes are most pronounced in the subendocardial region of the left ventricle and at the base of papillary muscles (Sisson & Thomas 1995). Darke and Else (1984) found canine myocardium grossly often appeared normal but there

were irregular patchy areas or occasional focal pale areas like distinct infarcts. Over 25% of dogs in atrial fibrillation had no obvious atrial lesions even on histopathology but in some cases fibrosis was present. These authors supported the findings of van Fleet and colleagues (1981) that disseminated foci myocardial necrosis, scattered areas of myocardial fibrosis and medial hyperplasia of intramyocardial arteries all were apparent in most cases. Darke & Else (1984) additionally reported fragmented muscle bundles together with lipoid droplets, myocytolysis and diffuse infiltration of collagen. Giant and irregular mitochondria with some bizarre mitochondrial figures were also recognised. Currently, however, the increased numbers of mitochondria and ultrastructural changes affecting mitochondria are generally regarded as nonspecific markers of cell damage (Sisson & Thomas 1995). Attenuated wavy fibres and interstitial fibrosis have been reported (Tidholm 1996; Tidholm & Jönsson 1996). These workers considered that the presence of attenuated wavy fibres, defined as myocardial cells less than six microns in width with a wavy appearance, were almost a pathognomic finding of canine DCM (Tidholm *et al* 1997; 1998a). They used the presence of attenuated wavy fibres to define the condition of occult DCM, prior to any clinical or echocardiographic abnormality (Tidholm *et al* 1998b).

Darke & Else (1984) felt that gross post mortem and light microscopy offered no further understanding as to the aetiology of DCM in the dog, especially as the histological and ultrastructural changes at post mortem are nonspecific and may be seen in other forms of advanced heart failure. This limits the usefulness of other proposed diagnostic techniques such as endomyocardial biopsy (Cobb 1992). Post-mortem specimens, rather than endomyocardial biopsy, were used in the attenuated wavy fibre studies of Tidholm and colleagues (1998a;b).

A.9. *Treatment of canine DCM*

Treatment is directed at alleviating the patient's clinical signs of heart failure and attempts to monitor and control arrhythmias (Keene 1989) with the goals of improving quality of life and prolonging survival (Sisson & Thomas 1995). Advances into the treatment have resulted primarily from research into therapy of

congestive heart failure rather than specific treatment of the diseased myocardium (Cobb 1992). A balanced pharmacological approach is normally used to optimise cardiac output by manipulating the major determinants of left ventricular performance, namely preload, afterload, contractility and controlling heart rate and rhythm (Fox 1988). The adverse neuro-hormonal consequences of congestive heart failure are also counteracted with therapy. Diuretics, the angiotensin-converting enzyme inhibitors and digoxin are indicated in most cases. Antiarrhythmic drugs may be indicated in some cases. Detailed description of the therapeutics of DCM is beyond the scope of this thesis.

A.9.1. *Angiotensin converting enzyme inhibitors*

The angiotensin converting enzyme (ACE) inhibitors, particularly enalapril, have become almost mandatory in the treatment of DCM after human trials (CONSENSUS (The CONSENSUS trial study group 1987) and SOLVD (The SOLVD investigators 1991)) and veterinary trials (IMPROVE (The IMPROVE study group 1995) and COVE (The COVE study group 1995)) have documented the efficacy of enalapril in improving quality of life and survival times in humans and dogs with DCM. It is a logical treatment as it counteracts the adverse neurohormonal activation seen in congestive heart failure.

As well as improvement in moderate and severe congestive failure, ACE inhibitors have been shown in man to reduce morbidity in patients with asymptomatic left ventricular dysfunction (The SOLVD investigators 1992). They may have the same beneficial effect in dogs with early DCM. This has been supported by the initial findings of O'Grady and colleagues (1997), comparing Dobermanns with occult DCM receiving an ACE inhibitor with those not on any therapy. The ACE inhibitor group showed increased time to onset of clinical signs.

A.9.2. *Nutritional Therapy*

Keene & Bonagura (1995) advocate trial therapy of L-carnitine for three months where owners of patients have no financial constraints at a dose of 220 mg/kg divided three times daily.

When DCM is identified in spaniels and small breeds of dogs, it is recommended that plasma or blood taurine levels are assayed to investigate whether taurine deficiency is an aetiological factor (Keene & Bonagura 1995). Taurine supplementation has also been recommended in breeds susceptible to urate or cysteine urolithiasis (Pion *et al* 1998), which includes the Newfoundland.

Nonspecific nutritional additives to attempt to improve myocardial function have also been advocated. Co-enzyme Q10 or ubiquinone (2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone) is a lipid soluble compound located on the inner mitochondrial membranes where it functions as an integral part of electron transport of oxidative phosphorylation. It is essential for the aerobic production of energy as ATP. Benefits have been reported in human idiopathic DCM but only after three to six months of supplementation (Langsjoen *et al* 1985; 1990a;b;c) and it has been advocated for canine idiopathic DCM at doses of 30 - 90 mg/day.

Some veterinarians or cardiologists advocate non-specific L-carnitine, taurine and co-enzyme Q10 supplementation in an attempt to improve myocardial function (Keene, 1991, Freeman *et al* 1996).

A.10. *Prognosis of DCM*

The prognosis for dogs with DCM is generally stated to be guarded or poor (Ettinger & Suter 1970; Wilkes 1980, Darke & Else 1984; Darke 1985; Ware & Bonagura 1986; Cobb 1992) with mean survival time of six months (Fox 1989). It is a relentlessly progressive disease (Calvert 1992) and after the onset of signs of congestive failure, the survival time is short, although prior to this, the dog may show no signs of the disease. Prognosis varies with breed. In Dobermanns, survival times

are short while cocker spaniels may live a long time (Calvert 1992; Darke *et al* 1993). There is no cure and there have been no reports of spontaneous recovery (Sisson & Thomas 1995). It is not possible to predict response to treatment or prognosis based on initial clinical parameters (Thomas 1987). An investigation into survival and prognostic indicators associated with this condition was undertaken by Monnet and colleagues (1995). Pleural effusion and pulmonary oedema were statistically significant independent prognostic indicators for survival. The median survival was only two months after initial echocardiography confirmed the diagnosis. However, dogs surviving in excess of seven months had a good probability of becoming long term survivors. The biphasic nature of this suggests that there may be disparity in the severity of disease at initial presentation, possibly due to variable owner awareness of clinical signs or to variable aetiology of the disease (Monnet *et al* 1995).

The risk of sudden death in DCM is noted in man. It can occur at any stage (Richardson *et al* 1996), as has been shown in dogs, particularly Dobermanns (Calvert 1995a).

A.11. *Occult dilated cardiomyopathy*

In canine cardiology, the term occult DCM was first coined and the course described by Calvert (1992), mainly referring to Dobermanns. He recognised that dogs with occult or early DCM have left ventricular dilatation and dysfunction beginning insidiously and progressing gradually over a period of several years and affected dogs exhibit no overt abnormalities prior to the development of congestive heart failure. Clinical examination may reveal no abnormalities. During the early decompensation stage (ejection fraction reduced by 35 - 50%), a murmur and / or S3 may be auscultable. Clinical signs of congestive heart failure or low cardiac output are not usually apparent until ejection fraction has reduced by 60 - 70% (Calvert 1995b).

In Dobermanns, Calvert (1992) described left ventricular dysfunction gradually progressing for two to two and a half years, after which left ventricular dilatation and

reduced contractility accelerates and congestive heart failure usually occurs within another year. Ventricular arrhythmias are evident throughout the course and sudden death may intervene at any time. Some dogs may develop DCM at older ages and abnormalities may not be detected until they are over ten years old. They may die of intercurrent disease and the DCM is never recognised (Calvert 1992).

Calvert (1995b) recognised that it was desirable to diagnose DCM during the occult / preclinical stage of the disease. By the time the dog shows clinical signs, the disease is advanced and therapeutic intervention at this stage is unlikely to significantly alter the course of the diseases. Selective screening for DCM is difficult as there may be no particular reason to suspect DCM other than breed predisposition but Calvert (1995b) recommends routine annual screening of dogs at high risk of developing DCM, particularly all Dobermanns and families within other breeds where familial disease is prevalent.

In the Dobermann and boxer, echocardiography and ambulatory ECG are useful in the diagnosis of occult DCM. Ventricular ectopy may precede echocardiographic abnormalities in the Dobermann by some months or over a year (Calvert 1995a). Diagnosis of occult DCM in breeds not characterised by arrhythmias is difficult (Calvert 1995b). Brownlie (1991; personal communication) indicated that conduction disturbances or atrial fibrillation precede the development of echocardiographic abnormalities or clinical signs in the Irish wolfhound with occult DCM.

Even in man, the diagnosis of occult DCM is controversial. Some relatives of DCM patients in Keeling & McKenna's (1994) series of human familial DCM were asymptomatic yet had increased left ventricular end-diastolic dimensions after correction for age and body size, some had audible diastolic gallops, some had decreased exercise capacity and some had late potentials on the signal averaged ECG. All these factors may indicate that these relatives have early DCM.

Treatment of occult DCM remains controversial and whether early medical intervention is able to influence the progression of left ventricular dysfunction is not proven. The prevention arm of the SOLVD trial of enalapril in human patients with asymptomatic DCM suggested that the onset of heart failure could be delayed (The SOLVD investigators 1992). However, it appears unlikely that ACE inhibitors will alter the progression of disease if they are used too early (prior to activation of the renin-angiotensin-aldosterone system) (Calvert 1992). If ACE inhibitors are used too early, tolerance may develop (Fox & Sisson 1995), and the initial early compensatory ventricular remodelling associated with tissue angiotensin II is inhibited, which may result in more rapid ventricular dilatation, as suggested by Häggström and colleagues in cavalier King Charles spaniels with mitral valve disease (personal communication; cited by Wotton 1998a). ACE inhibitors are indicated once evidence of early decompensation is present (Calvert 1995b) and appear to slow progression to the onset of clinical signs in Dobermanns with occult DCM (O'Grady *et al* 1997).

A.12. *Breed specific forms of idiopathic DCM*

DCM has been reported in a variety of breeds and breed predispositions lend support to a genetic basis to this condition. Bulldogs were reported in the early literature (Ettinger & Suter 1970; Ettinger 1975; Ogburn 1977) and immunological basis to myocarditis present in Bulldogs has been postulated (Ware & Bonagura 1986) although a non-inflammatory "myocardiopathy" in a Bulldog was reported by Otter (1991). German Shepherd dogs have been included in the earlier literature as being commonly affected (Ettinger & Suter 1970, Ettinger 1975; Ogburn 1977) although the breed is currently under-represented as having the condition (Tidholm, 1996). Dalmations have recently been reported in a series by Freeman and colleagues (1996), but in association with urate urolithiasis (Pion *et al* 1998). Weimaraners have not been reported in the literature, although were recognised during the course of this study (data not shown). A juvenile onset malignant dilated cardiomyopathy has recently been reported in Portuguese water dogs (Dambach *et al* 1999).

DCM has been reported in a variety of breeds but the best characterised phenotypic forms of the condition reported appear to be distinct for the giant breeds (Newfoundlands, great Danes and Irish wolfhounds), Dobermanns, boxers and cocker spaniels.

A.12.1. *Newfoundlands*

Newfoundlands were included in the list of breeds said to be predisposed to DCM in the earliest reports of the canine condition (Ettinger & Suter 1970). Sisson and Thomas (1995) reported the prevalence of DCM in this breed in the U.S. as 1.3%. In a series of dogs with atrial fibrillation (Bohn *et al* 1971), breed specific prevalence rates were reported. Newfoundlands had the highest rate of 56.6/1000 (although numbers were small; there were 53 Newfoundlands out of a population of over 30 000 dogs, of which three Newfoundlands had atrial fibrillation although the numbers of Newfoundlands with cardiac disease was not reported). Although the associated underlying heart disease was not reported for Newfoundlands specifically, it is interesting to speculate that some of these cases may have been due to DCM, although subaortic stenosis was listed as being associated with atrial fibrillation and Newfoundlands were later reported by some of these authors as being predisposed to this condition (Pyle *et al* 1976; Jones *et al* 1982).

The features of DCM in Newfoundlands are consistent with those reported for the giant breeds in general. Tidholm and Jönsson (1996) reporting on 37 Newfoundlands with DCM found no statistically significant sex predisposition (more bitches were included in their series). The age range of dogs affected with DCM and congestive failure was 3.5 months to 11.7 years with a mean and median of five years. These authors noted that many of these dogs were hyperthermic. Murmurs associated with DCM were reported as being infrequent, although the authors were aware that they may have missed murmurs as they are generally low grade, the dogs panted and adventitious respiratory sounds may have masked the murmurs. No mention was made of the conformation and hair-coat further making this breed difficult to auscultate.

A.12.2. *Great Danes*

Great Danes were reported as being a breed predisposed to DCM in the earliest veterinary literature describing the canine condition (Ettinger & Suter 1970; Ettinger 1975; Ogburn 1977) and are commonly included in series of cases (Monnet *et al* 1995). Prevalence rates in Great Danes in the U.S. are reported to be 3.9% with age dependant prevalence reported (Sisson & Thomas 1995). An affected dog as young as 0.33 years has been reported (Fleet *et al* 1981), and there has been confusion in the literature between mitral insufficiency, mitral incompetence, DCM and what has since been recognised as mitral dysplasia in the breed (Hamlin *et al* 1965; Hamlin & Harris 1969; Dear 1971). In 1971, Great Danes were recognised as being predisposed to atrial fibrillation (Bohn *et al* 1971; Boevé *et al* 1984) and also show ventricular arrhythmias (Hamlin & Harris 1969, Dear 1971), associated with sudden death. Great Danes were over-represented in a pathological study of DCM (van Fleet *et al* 1981).

A.12.3. *Dobermanns*

Dobermanns were not listed in the original report of canine DCM affected breeds (Ettinger & Suter 1970), but this breed features prominently in subsequent reports (van Fleet *et al* 1981; Calvert *et al* 1982; Hazlett *et al* 1983; Calvert 1984; Lombard 1984a; McCarthy 1984; Darke & Else 1984; Calvert 1986; Monnet *et al* 1995). Congestive heart failure and sudden death has been recognised in Dobermanns in the USA since the early 1950s (Calvert 1995a). Pedigrees of Dobermanns demonstrate that cardiac disease can be traced back to the original members of this breed in North America (Meurs *et al* 1996b). Sisson and Thomas (1995) report the prevalence of DCM in this breed to be 5.8%. Other authors report much higher rates, with 63.2% of Dobermanns in a longitudinal study of 192 initially normal dogs affected by 4.5 years follow up (O'Grady & Horne 1992; 1998). The prevalence increases, ranging from 0.29% in Dobermanns less than one year old to 10.6% in Dobermanns older than ten years (Sisson & Thomas 1995). The age of onset of congestive heart failure due to DCM is on average $7^1/_2$ years old in males and $8^1/_2$ - 9 years old in females (Calvert 1995a).

Dobermann DCM is strongly suspected to be a familial disease. Since Wood (1983) first proposed this, increasing evidence has supported this view, with reports of family clusters with DCM affecting four consecutive generations (Calvert 1992; 1995a). In some kindreds, an autosomal dominant mode of inheritance is suspected (Meurs 1998).

Most veterinary cardiologists consider that Dobermanns have a more severe and rapidly progressive form of DCM than other breeds (Wood 1983; Ware & Bonagura 1986; Keene 1989; Calvert *et al* 1997b), although Monnet and colleagues (1995) failed to demonstrate statistically that this breed was less likely to survive. Many Dobermanns present with apparently acute onset disease, with fulminant pulmonary oedema. However, serial evaluation in longitudinal studies indicate that DCM is in fact a chronic, slowly progressive disease (Calvert 1992;1995a; Calvert *et al* 1997a). Adverse prognostic indicators for survival in symptomatic Dobermanns include the presence of biventricular failure and atrial fibrillation in this breed (Calvert *et al* 1997a). Sudden death may occur at any stage of the development of DCM, although dogs with periods of sustained ventricular tachycardia were at increased risk (Calvert *et al* 1997b).

A number of myocardial biochemical abnormalities have been associated with Dobermann DCM (McCutcheon *et al* 1992; O'Brien *et al* 1992;1995; O'Grady *et al* 1992; O'Brien 1997), as summarised previously (Section A.4.1.), consistent with impaired oxidative phosphorylation. Dobermanns with proven myocardial carnitine deficiency survive longer with supplementation and conventional treatment than non-deficient dogs (Keene *et al* 1989; Keene 1991).

A.12.3.1.

Dobermann occult DCM

Smucker and others (1990) noted that even apparently normal Dobermanns had occult left ventricular dysfunction. Calvert (1992; 1995a) published observations of

great value about the preclinical (occult) stages of DCM in this breed. In general, the disease begins in dogs between three to six years of age, with death due to congestive heart failure occurring three to four years later, although sudden death can occur at any time. Survival times are short once congestive heart failure develops (Calvert 1995a). After onset of clinical signs of congestive heart failure, mean survival time is reported as 9.5 weeks with a median of five weeks (Calvert 1992).

Calvert (1995a) reported that 25 - 30% of asymptomatic Dobermanns between the ages of two and 14 years old show echocardiographic or Holter evidence of DCM. It is important that dogs are identified prior to anaesthesia for surgical procedures as exacerbation of arrhythmias or systolic dysfunction may be life threatening (Calvert *et al* 1996).

When Dobermanns developing DCM are followed sequentially, progressive left ventricular dilatation and dysfunction occurs over a period of 2 - $2^1/_2$ years and then accelerates resulting in congestive heart failure in another year, although some Dobermanns may show more slowly progressive disease (Calvert 1992; 1995a). If abnormalities are evident in Dobermanns as pups or at less than two years of age, they tend to show an accelerated course. If Holter or echocardiographic abnormalities are not detected until dogs are greater than six years old, the course is much more prolonged (Calvert 1995a). When left atrial enlargement is radiographically evident, Dobermanns with occult DCM will generally develop left sided heart failure within nine months (Calvert 1992).

Calvert (1995a) gave some criteria for the Holter diagnosis of occult DCM based on the frequency of ventricular ectopy in Dobermanns. Dobutamine stress echocardiography has also been utilised in the identification of very early systolic and diastolic dysfunction which herald the development of occult DCM (Minors & O'Grady 1998).

Ventricular arrhythmias are believed to be responsible for sudden death in most Dobermanns with DCM, which can occur at any stage. Up to 25% of Dobermanns with occult DCM die suddenly while the remaining 75% develop congestive heart failure (Calvert 1995a). The mean age for sudden death associated with DCM is reported as $6^1/_2$ - 7 years old with a male predisposition and 20% of Dobermanns with DCM (all stages) die suddenly (Calvert 1992). Not all syncopal Dobermanns with DCM collapse due to ventricular arrhythmias. Calvert (1995a) reported that cardiomyopathic Dobermanns were prone to neurocardiogenic syncope associated with bradycardia on excitement or exertion.

A.12.3.2.

Echocardiographic screening

In order to identify echocardiographic abnormalities indicative of occult DCM, reference ranges of normality are required. This is discussed in more detail in Section B of this thesis. Briefly, however, normal Dobermanns up to 45kg body weight recognised by Calvert (1995a) have fractional shortening of 30 - 36%, left ventricular end-diastolic dimensions less than 50 mm, interventricular septal and left ventricular free wall systolic dimensions exceeding 10 mm and diastolic dimensions greater than 7 mm. Calvert (1995a) reported that a fractional shortening < 25% was abnormal. In Dobermanns identified with occult DCM, dogs with fractional shortening less than 20% develop congestive heart failure within one year.

The older a Dobermann is with unremarkable echocardiographic and Holter findings, the more likely it is to be truly normal, although Calvert (1995a) urged extreme caution in declaring any Dobermann to be normal and felt that the only conclusion may be that it would not die prematurely due to DCM. It is likely that a great number of Dobermanns are affected but the disease may be sufficiently mild in some dogs to allow them to achieve advanced age before developing overt disease and may die of intercurrent disease before any cardiac signs are appreciated (Calvert 1995a).

A.12.4. *Cocker spaniels*

Cocker spaniels (probably the American cocker spaniel) affected with congestive heart failure and particularly males were recognised to be over-represented during screening dogs to assess the prevalence and types of cardiovascular disease between 1948 and 1965 (Detweiler & Patterson 1965). Sisson & Thomas (1995) recorded the U.S. prevalence of DCM in English cocker spaniels as 0.69% and in American cocker spaniels as 0.34%. Wotton (1996b) estimated a UK prevalence of DCM in cocker spaniels as 3.4%. Wotton (1996b) presented the data from 27 cocker spaniels of ages 3.5 months to 11 years (mean 4.6 $\pm$ 2.6 years) with males slightly over-represented (59%). Two spaniels (unspecified) were reported in the series of 50 cases by Darke and Else (1984). Cocker spaniels (presumably American) were included in the series by Monnet *et al* (1995).

The first report of English cocker spaniels with myocardial disease was from an isolated Australian kennel (Staaden 1981). A familial basis to this condition was recognised. Another Australian paper presenting material from the same kennel (Gooding *et al* 1982) reported findings from 49 dogs, 26 of which were normal, and proposed that concentric hypertrophy progressed to eccentric hypertrophy (i.e. hypertrophic cardiomyopathy progressed to dilated cardiomyopathy). In a later publication, however, Gooding and colleagues (1986b) did not mention concentric hypertrophy further but they presented an echocardiographic characterisation of DCM in cocker spaniels from this kennel. However, the control group was selected from the same kennel and five dogs were shown to have reduced fractional shortening. These findings may suggest that this subgroup had occult DCM and that the echocardiographic abnormalities precede electrocardiographic, radiographic and clinical abnormalities. The first report in the UK of this condition was by Thomas (1987a) who documented presumed congestive cardiomyopathy in eight cocker spaniels in the UK, six of whom were related.

These initial reports suggesting that some cockers have concentric hypertrophy and possibly hypertrophic cardiomyopathy is confusing. In the absence of definitive data

documenting progression, it seems unlikely that these cases represent the same condition. A case of presumed hypertrophic cardiomyopathy in a cocker spaniel documented by echocardiography was reported by Wotton (1996b; 1998d).

Cocker spaniel DCM has a more benign course than other breeds, with some dogs surviving several episodes of severe congestive heart failure (Gooding *et al* 1986; Wotton 1992; 1996b;1998c;d). Arrhythmias are uncommon, although pulsus alternans is recognised in some cases (Staaden 1981; Wotton 1996b).

Keene and Bonagura (1995) recommend that spaniels with DCM should have blood or plasma taurine levels assayed to determine whether taurine deficiency is an aetiological factor. American cocker spaniels are known to respond incompletely to taurine and L-carnitine supplementation (Kittleson *et al* 1997). Wotton (1996b; 1998d) felt that there was no evidence that taurine or carnitine deficiency was a significant cause of heart disease in the UK.

A.12.5. *Boxers*

Boxers first reported in 1970 as being susceptible to DCM (Ettinger & Suter 1970). By 1983, the fact that Boxers had a breed-specific form of DCM was recognised (Wood 1983) and Harpster (1983) gave an excellent clinical description of the condition, which is still utilised today (Wotton 1996a;1998b). The prevalence of DCM in Boxers in the US has been reported as 3.4% with increased prevalence associated with age (Sisson & Thomas 1995). DCM in UK Boxers is not identical to that occurring in the USA, as the arrhythmic forms are less prevalent (Wotton 1996a). Even in the USA, there may be geographical differences in the distribution and clinical characteristics (Keene 1989).

Harpster (1983) recognised that there was less marked ventricular dilatation in the Boxer compared with other breeds with DCM and atrial fibrillation was infrequent. Harpster (1983) reported on 64 Boxers which were closely related and an inherited basis to the disease was suspected; some family lines had greater prevalence of

disease. The survey of heart disease in Boxers was updated to a total of 112 dogs (Harpster 1991). Males were slightly over-represented (56.3%) and Boxers of one to fifteen years of age years were affected, although most Boxers were over six years old with a mean age of 6.9 years (Harpster 1991). Arrhythmias (usually ventricular) are common and murmurs of mitral regurgitation secondary to dilatation of the atrioventricular annulus occur later in the course of the disease. Diastolic gallops are frequent once congestive failure has developed. An electrocardiogram is said to be almost pathognomic with ventricular premature complexes or runs of right ventricular origin (left bundle branch block pattern) (Harpster 1983; 1991).

Harpster (1983; 1991) recognised three categories to DCM resulting in different presentations of the boxer breed. In category 1, dogs are asymptomatic but an arrhythmia may be detected. Holter monitoring is used to identify dogs (Meurs & Brown 1998). In category 2, affected dogs have a history of episodic weakness or collapse. Some degree of cardiomegaly may be identified. Category 3 is characterised by the presence of congestive heart failure.

Pathological findings are distinct in this breed. Histologically, there is usually evidence of active or chronic changes. The right ventricle is the earliest and the most severely affected (Harpster 1991). Active changes described included focal areas of myocytolysis, myofibrillar necrosis, haemorrhage, a mild cellular infiltrate (predominantly mononuclear cells) and the chronic changes included myofibrillar atrophy, marked variation in size and shape of individual fibres, fibrosis and extensive fatty infiltration and fatty change (Harpster 1983; 1991). The characteristic pathology associated with Boxer cardiomyopathy, in contrast to the non-specific changes recognised in other breeds (Sisson & Thomas 1995) and man with DCM has led some authors to refer to the condition as Boxer myocarditis (Tidholm *et al* 1998a). However, the histological findings are very similar to those in human arrhythmogenic right ventricular cardiomyopathy (also called arrhythmogenic right ventricular dysplasia, ARVD), particularly with respect to the fatty infiltration.

Wotton (1998b) has proposed that boxer cardiomyopathy bears marked resemblances to the human condition.

Keene and colleagues (1991) recognised that myocardial L-carnitine deficiency was associated with DCM in a family of Boxers, with improvement demonstrated after L-carnitine supplementation in many but not all dogs (Keene 1991). Even when effective, L-carnitine supplementation does not influence ventricular ectopy and antiarrhythmic medication is indicated (Harpster 1991; Keene 1991; Meurs & Brown 1998).

A familial basis to the disease was suspected by Harpster (1983) in his original report. The familial dysrhythmia is reported to be transmitted as an autosomal dominant trait is some families (Meurs *et al* 1998), although Wotton (1998b) felt that an autosomal recessive mode of inheritance was more consistent with pedigree data from his study.

A.12.6. *Irish Wolfhounds*

Irish wolfhounds were first reported in 1970 as being susceptible to DCM (Ettinger & Suter 1970; McCarthy, 1984; Thomas 1987a). Irish Wolfhounds were reported as having a high prevalence rate of atrial fibrillation (41.6/1000) (Bohn *et al* 1971) and it is likely that many of these cases were due to DCM although this disease was not mentioned in the text. Atrial fibrillation and other electrocardiographic abnormalities were reported to precede the development of occult DCM in this breed (Brownlie 1991). Similar electrocardiographic abnormalities were reported in European dogs (Vollmar 1996). The prevalence rate from a group of 440 dogs was given as 16.4%, with abnormal electrocardiographic findings in 34.1% of dogs (Vollmar 1998). Brownlie and Nott (1991) demonstrated that males with larger girth measurements and females with increased girth and height measurements had a higher incidence of heart disease. In one line of Irish Wolfhounds, an inherited basis of the disease has been shown, with probable autosomal dominant mode of inheritance (Cobb *et al* 1996).

SECTION B

ECHOCARDIOGRAPHIC / DOPPLER DIAGNOSIS OF DILATED CARDIOMYOPATHY

A REVIEW OF THE LITERATURE

B.1. *Assessment of Chamber size*

B.1.1. *Introduction*

Dilated cardiomyopathy (DCM) is characterised by dilatation and impaired contraction of the left ventricle (Richardson *et al* 1996). Diastolic abnormalities are increasingly recognised with this condition (Werner *et al* 1994; Fruhwald *et al* 1997b). It therefore follows that echocardiographic criteria which identify left ventricular dilatation and impaired systolic and / or diastolic function of the left ventricle may be used to diagnose this condition. Echocardiography / Doppler is also an excellent modality to exclude most primary cardiac causes of secondary myocardial failure. A knowledge of normal chamber size and reference parameters for criteria of systolic and diastolic function determined by echocardiography / Doppler is required to identify early abnormalities associated with the development of DCM. Breed specific reference ranges are required. Although there are some reference ranges for echocardiographic parameters published in man, measurements outside these reference ranges are difficult to evaluate and Vasan and colleagues (1997) proposed partitioning abnormal echocardiographic measurements, normalised to height and gender, by their deviation from the reference using 95 - 99% percentile values. A large number of echocardiographic parameters are available in the assessment of specific chamber size and systolic and diastolic function and these will be reviewed.

B.1.2. *Assessment of left ventricular size*

There are a number of methods for estimating left ventricular size or volume. M-mode echocardiography has conventionally been used to measure the diameter of the left ventricular lumen as well as the interventricular septum and left ventricular free

wall thickness. In veterinary echocardiography, the only method of quantifying left ventricular function has tended to be by M-mode echocardiography until recently. In veterinary echocardiography, most cardiologists follow the American Society for Echocardiography recommendations (Sahn *et al* 1978). However, these M-mode echocardiographic recommendations are the result of a questionnaire to echocardiographers cardiologists with a poor response rate (19%) (Sahn *et al* 1978; Luis Fuentes & Bonagura 1998). They were, however, largely based on measurement techniques with the least coefficient of variation (standard deviation divided by mean) between observers measuring the same brief M-mode echocardiogram. End-diastole is defined as the start of the QRS complex and systole as the nadir of the septal systolic posterior motion where septal motion is normal (Sahn *et al* 1978). Since most M-modes obtained today are guided using a cursor on an optimised two-dimensional image, it is now advised that a short axis two-dimensional view from a right parasternal window should be used to place the M-mode cursor at the level of the chordae tendinae (Feigenbaum 1994a). The leading edge-leading edge technique should be used as the M-mode endocardial echos may be affected by gain settings (Sahn *et al* 1978; Feigenbaum 1994a) although this is not always done. O'Grady *et al* (1986) and Allworth *et al* (1995) used intraluminal left ventricular measurements i.e. trailing edge to leading edge. Positioning of the M-mode cursor within the left ventricular cavity is critical, and prior orientation with both the long axis and short axis cross sectional images is essential for successful placement (De Madron 1995; 1996). The long axis image is used to ensure that the left ventricular axis is perpendicular to the M-mode cursor, and that both the inter-ventricular septum and left ventricular free walls are transected at similar levels, at the level of the chordae tendinae. The short axis view is then selected to ensure that the M-mode cursor bisects the centre of the left ventricular cavity.

In man, the left ventricular length and diameter show a strong linear relationship with height, weight and body surface area (BSA). However, if data are corrected for height, body weight and BSA have no additional effect on parameters of left ventricular size (Weyman 1994b). The parameters of left ventricular internal

diameter in diastole (LVIDd) and systole (LVIDs) are affected by heart rate (Jacobs & Mahjoob 1988a;b) and are also highly preload, afterload and contractility dependent (Borow 1989).

Calculations of left ventricular volume from the minor axes have been described, the simplest of which is the cubed formula for a prolate ellipse:

$$\text{Volume} = (\pi/3)\text{x}(\text{LVID})^3$$

Although this formula is reasonably accurate in normal ventricles, it becomes increasingly inaccurate in dilated ventricles as they assume a more spherical rather than an elliptical shape (and the major axis becomes less than twice the minor axis) (Movsowitz *et al* 1996). The Teicholz correction for the decrease in the major: minor axes ratio associated with left ventricular dilatation is more accurate. The Teicholz formula is shown (Borow 1989) (Movsowitz *et al* 1996):

$$\text{Volume} = [7/(2.4 + \text{LVID})]\text{x}(\text{LVID})^3$$

It should be noted that M-mode derived left ventricular volumes are based on geometrical assumptions which become invalid in the presence of regional wall motion abnormalities or altered left ventricular morphology.

These techniques have been largely superseded by two dimensional calculations of left ventricular volume in human cardiology although veterinary texts including ventricular volume and ejection fraction usually refer to M-mode derived parameters (Freeman *et al* 1996). However, left ventricular diameters have a wide range of error for the assessment of left ventricular size, especially for enlarged ventricles and left ventricular volumes calculated by two dimensional echocardiography is the preferred method to assess left ventricular remodelling (Dujardin *et al* 1997). In addition, M-mode echocardiography only measures a small point of the left ventricle at a single level of the interventricular septum and left ventricular freewall. Various two-

dimensional methods of assessing left ventricular volume, and so global left ventricular function, have been devised (Feigenbaum 1994a; Vuille & Weyman 1994).

The geometrical assumption that the left ventricular lumen approximates the shape of a prolate ellipse has led to a variety of calculations of left ventricular volume based on this mathematical model (Movsowitz *et al* 1996). The length-area method calculates left ventricular volume from a single plane, conventionally done from the apical four chamber view (Borow 1989; Schiller *et al* 1989; Feigenbaum 1994a; Vuille & Weyman 1994).

$$\text{Vol} = \frac{8 \times (\text{area}^2)}{3 \times \pi \times \text{length}} = 0.85 \times \frac{\text{area}^2}{\text{length}}$$

The single plane ellipse method may also be determined from a two chamber view or apical long axis view of the left ventricle. The left ventricular length and volumes are larger from the four chamber view (St. John Sutton *et al* 1998). The formula may be modified for use with two orthogonal planes (i.e. from left apical two-chamber and four chamber views).

One of the problems in serial evaluation of changes in load are the potential consequences on geometrical assumptions. Gaynor and colleagues (1994) found that a cylindrical model was most accurate at estimating changes in volume associated with differing loads, although this model did not estimate true left ventricular volume accurately. Eaton and colleagues (1979) showed that serial cross sectional views of the left ventricle every three millimetres could be used to accurately assess volume. These authors had the foresight to predict that with advances in computer technology and echocardiography that this would become a viable technique and, indeed, this has become the basis of Simpson's method to estimate left ventricular volumes.

One of the most accurate methods of assessing left ventricular volumes is Simpson's rule, which has now evolved to the modified Simpson's rule (the biplane method of

discs) (Borow 1989; Schiller *et al* 1989; Feigenbaum 1994a; Schiller & Foster 1996). This is based on the mathematical principle that the sum of volumes of multiple slices of known thickness through an object equal the total volume of the object. This formula is consequently independent of any geometrical assumptions and it correlates well with angiographically derived left ventricular volumes (Movsowitz *et al* 1996; Schiller & Foster 1996). In echocardiographic software, twenty slices are used. Volumes and ejection fraction can be reliably determined even in the presence of regional wall motion abnormalities (Albin & Rahko 1990). The disadvantage of Simpson's rule is that at least two image planes are required (a two chamber and four chamber left apical view) and it is a time consuming technique even with echocardiographic software. Nosir and others (1997) reported that an apical long axis view rather than the two chamber view should be used with the four chamber view, as it was more truly orthogonal and it was easier to standardise the view as the valve leaflets of the mitral and aortic valves provided landmarks for good central alignment.

Obtaining the area measurements is fraught with problems as the lateral resolution may not be good and endocardial images parallel with the imaging ultrasound beam suffer from echo dropout making accurate tracing of endocardial surfaces prone to error. In addition, as the area measurement is squared, any measurement error in this parameter is multiplied four-fold. It is because of this problem that the American Society of Echocardiography suggest that two-dimensional echocardiographic measurements should be made from "black-white" interfaces, i.e. a trailing edge to leading edge (Schiller *et al* 1989). In people, two-dimensional imaging may be too poor to allow determination of volumes in ten to twenty percent of individuals, and there is substantial inter- and intra-observer variability in the measurements (Movsowitz *et al* 1996). Echocardiography consistently underestimates left ventricular volume (Vuille & Weyman 1994). Increasingly, on sophisticated machines, software to improve endocardial boundary detection is being used (Movsowitz *et al* 1996). This technique, known as acoustic quantification, is based on ultrasonic backscatter analysis and it allows on-line continuous display of

endocardial borders which allow beat to beat calculation of cavity areas, volume and ejection fraction (Gorscan *et al* 1993b; Yvorchuk *et al* 1994) and stroke volume (Gorscan *et al* 1993a). It has been validated in canine open chest models (Morrissey *et al* 1994). The technique has also been used to estimate left ventricular compliance from end-diastolic pressure / volume curves (Gorscan *et al* 1994). There is a report of acoustic quantification being assessed in dogs and cats (Rosenthal & Saunders 1996).

Although it is tempting to assess left ventricular area from a right parasternal long axis four chamber view, because of improved endocardial delineation as the images are perpendicular to the ultrasound beam, this plane fails to include the left ventricular apex (Vuille & Weyman 1994) and there have been no reports or validation of using this view in human echocardiography. However, attempts at obtaining a left apical view in dogs have not been successful (Thomas 1984; Thomas *et al* 1993). O'Grady and colleagues (1986) found that the longest left ventricular length was obtainable from the right parasternal view and proposed that the right parasternal view may be more accurate for assessing left ventricular volumes in dogs. The left ventricular volumes (calculated by length / diameter calculations) reported by these authors were obtained from an RPS view (O'Grady *et al* 1986). It was felt that left apical views could not be adequately obtained in dogs because the cardiac window (between lung lobes) was smaller than on the right hemithorax (O'Grady *et al* 1986).

The use of left ventricular volumes indexed to body weight or BSA has allowed comparison between individuals of differing sizes. The end-systolic volume index (ESVI) is recommended as a method of assessing systolic function in man, which is more reliably predictive of prognosis than the ejection fraction (Schiller & Foster 1996). The end-diastolic volume index (EDVI) is a valuable but less powerful predictor of cardiac events in man. Reference values are given for people for these indices by Schiller and Foster (1996). In humans, there is a gender difference in the normal EDVI and ESVI. In dogs, Amberger and Lombard (1998) gave figures for a

normal left ventricular end-systolic volume index (ESVI) as <30 mls/m^2 and indicated that ESVI >80 mls/m^2 was consistent with cardiac insufficiency, using the cubed or Teicholz formula from M-mode parameters. Dogs with DCM had a significantly decreased survival time if they had an end-diastolic volume index of > 210 mls/m^2 or with end-systolic volume indices > 138 mls/m^2 (Borgarelli *et al* 1998) which implies that the more dilated the ventricle, the worse the prognosis.

Cardiomyopathy without significant or only mild left ventricular dilatation has been described in the human literature (Keren *et al* 1988a; D'Cruz *et al* 1992). These patients had improved course and survival compared with classical DCM patients (Keren *et al* 1988a). Despite failing to meet the classically accepted criteria for the diagnosis of DCM, left ventricular shape, assessed by various methods comparing short axis with long axis dimensions was significantly more spherical than normal, age matched individuals, despite being slightly less spherical than the LV of patients with classical DCM (D'Cruz *et al* 1992). Douglas and colleagues (1989) also showed that LV shape was important. In human patients with DCM, survival was adversely affected by having a more spherical LV, with increased minor axis dimension, and more evenly distributed wall stress (assessed by the ratio of meridional to circumferential wall stress).

There are formulas for determining left ventricular mass, which have been validated anatomically (Devereux & Reichek 1977), although repeatability is poor (Kuecherer *et al* 1991). Stöllberger and others (1996) used a mathematical formula to show that even small measurement inaccuracies, in the order of 5%, a magnitude error inherent in echocardiographic measurements, result in changes in estimation of left ventricular mass by the Devereux formula of 8 - 15%. The measurement of left ventricular mass is described by Levy and Lauer (1996), with formulae derived from M-mode parameters.

B.1.3. *Assessment of left atrial size*

Left atrial size can be calculated by a number of techniques. From the right parasternal long axis four chamber view, the width of the left atrium just above and parallel to the mitral annulus and its length may be measured. The maximal left atrial dimensions and area are at the end of ventricular systole (Weyman 1994a) Similar measurements are usually obtained from an apical four chamber view, although the posterior wall is prone to echo dropout, but this is the standard view in human echocardiography (Weyman 1994a). Area measurements have also been described and may be improved by automatic boundary detection (Clarkson *et al* 1995). In dogs, O'Grady and colleagues (1986) assessed left atrial area by planimetry from the right parasternal views and an apical views and have found them to be highly correlated. Left atrial area and anterior-posterior and apico-basilar distances were correlated with body weight and BSA (O'Grady *et al* 1986). In man, area and diameters of the left atrium show a strong relationship for height. If corrected for height, the other variables, weight and BSA, have no other significant effect on left atrial parameters (Weyman 1994b).

A diastolic emptying index has been proposed by Clarkson and colleagues (1995) as:

$$\frac{\text{maximum atrial area - minimal atrial area}}{\text{maximum atrial area}}$$

Maximal left atrial area is at the end of ventricular systole. Atrial systole gives the smallest left atrial area (corresponding to the end of ventricular diastole - start of QRS complex on ECG). This measurement may also be considered as a derivative of a left atrial ejection fraction and intrinsic left atrial dysfunction has been shown in DCM in human patients (Ito *et al* 1996). The actual timing of maximal and minimal left atrial areas is controversial (Ito *et al* 1996). Volume calculations for the left atrium are not well validated (Vuille & Weyman 1994).

The left atrium has often been compared with the aortic root diameter at the level of the aortic valves in man and dogs. This may be done from a five chamber long axis view, which visualises the left auricular appendage more than the left atrial body or,

more conventionally, from an M-mode of the aortic root and left auricular appendage (Bonagura *et al* 1985). The aortic root is measured in diastole and the LA from ventricular systole and expressed as a ratio (LA:Ao).

In dogs, because of various chest conformations, by placing an M-mode cursor through the centre of the aortic root, the more distal intersection of the cursor can be variable and inconsistent; it may be different areas within the left auricular appendage or the left atrium or even the pulmonary trunk (De Madron 1995). To avoid this variability, it has been suggested obtaining the left atrial length from a short axis view, to its maximum length, in a plane which also intersects the aortic root (Häggström *et al* 1994). Both parameters are measured from two dimensional diastolic images and a ratio can be expressed .

Assessment of left atrial function is important to explain some of the features of pulmonary venous flow and it may be impaired in DCM (Ito *et al* 1996). In human essential hypertension, in patients with normal left ventricular systolic function, the left atrial size, particularly in systole, correlates well with LV wall thickness, better than the indices of diastolic function and Simek and others (1995) proposed that left atrial systolic size, in the absence of atrial fibrillation or mitral valve disease, indicated the severity and chronicity of left atrial hypertension. Left atrial size assessment was postulated by these authors to provide a single, non-invasive estimate of the degree of diastolic dysfunction. A left atrial area in man exceeding 18 cm^2 is an independent predictor of an adverse prognosis in patients with systolic failure (Giannuzzi *et al* 1996). Increased left atrial size and reduced function are strong negative predictors of exercise capacity (Triposkiadis *et al* 1992).

Left atrial size affects mitral annulus size independent of left ventricular dimensions. Consequently, isolated left atrial enlargement may result in mitral regurgitation despite normal left ventricular size and function and intrinsically normal mitral valve leaflets (Tanimoto & Pai 1996).

B.2. *Echocardiographic / Doppler assessment of left ventricular systolic function*

Left ventricular systolic function is determined by the extent of left ventricular fibre shortening during systole and is a result of the interaction between preload, afterload, heart rate and myocardial contractility (Movsowitz *et al* 1996). Contractility as an isolated parameter cannot be easily measured because of the inter-dependence on preload, afterload and heart rate.

B.2.1. *Fractional shortening (FS%)*

Fractional shortening, usually expressed as a percentage, is obtained from the LV M-mode measurements described above:

$$FS\% = \frac{LVIDd - LVIDs}{LVIDd} \times 100$$

(Allworth *et al* 1995).

This measurement relies on the minor axis of the left ventricle representing overall LV function (Vuille & Weyman 1994). It is predominantly preload, afterload and contractility dependent (Borow 1989). Lonsdale and colleagues (1998) showed that fractional shortening was influenced by training in greyhounds; it was increased in trained dogs.

There is considerable controversy in the veterinary literature about the lower reference limit for fractional shortening in normal dogs. Reference values have been generated in populations where DCM is prevalent, probably including a mixture of normal and cardiomyopathic individuals, and where follow up has been limited to two to three years at the most (Kienle 1998). Sarcomeres normally contract 30% (from diastolic length of 2.2 μm to systolic length of about 1.5 μm), which Kienle (1998) argued should result in a 30% change in left ventricular circumference and consequently a 30% change in left ventricular diameter.

B.2.2. *Percentage thickening of the interventricular septum (%thIVS) & left ventricular posterior wall (%thLVpw)*

These parameters are also obtained from the LV M-mode parameters and are calculated by the software of the echocardiograph machine (O'Grady *et al* 1986; Borow 1989; Allworth *et al* 1995):

$$\frac{IVSs - IVSd}{IVSs} \times 100\% \qquad \frac{LVpws - LVpwd}{LVpws} \times 100\%$$

Although usually systole is defined as the nadir of septal motion, sometimes the posterior wall (also called the free wall) lags behind this so a true "systolic" free wall measurement is not obtained, although most echocardiograph software prompts the measurement to be made in a line defined by systole. Allworth and colleagues (1995) used the LVpw systole as the peak anterior systolic motion of the free wall to obtain this measurement.

Wall thickening is almost linear throughout systole, although wall thinning is biphasic, with an initial rapid phase during the first 40% of diastole and a slower phase in late diastole. In DCM, the systolic wall thickening is similar but of lower magnitude, although the biphasic diastolic phase is lost and thinning is slowed (Goldberg 1984).

B.2.3. *Ejection fraction (EF)*

Calculation of the ejection fraction, usually expressed as a percentage, is based on determination of the left ventricular volumetric measurements.

$$EF = \frac{LVdv - LVsv}{LVdv} \times 100\%$$

The ejection fraction will give a better estimate of global left ventricular function, as during the fractional shortening method, only two small segments of myocardium are used. However, the ejection fraction reflects a complex interaction between preload, afterload and heart rate as well as contractility (Borow 1989). In man, the reference ejection fraction is 61 $\pm$ 10 % (Schiller & Foster 1996). Two dimensional

echocardiographic determination of ejection fraction has been appreciated to have high sensitivity and specificity for impaired left ventricular function since 1984 (Erbel *et al* 1984).

Errors in this measurement will be the same as are incurred during the volumetric measurements, although presuming the errors affect diastole and systole to the same degree, they are cancelled out (Borow 1989).

Many human cardiologists will visually estimate the ejection fraction fairly accurately ("eye-balling") (Movsowitz *et al* 1996; Schiller & Foster 1996). In additon, Silcocks and colleagues (1997) showed that a qualitative echocardiographic assessment of left ventricular dysfunction was of prognostic value, whereas no single quantitative echocardiographic index was of independent prognostic significance.

Determination of left ventricular diastolic (LVdv) and systolic volumes (LVsv) enables the stroke volume (SV) to be calculated, and therefore the cardiac output (CO), if the heart rate (HR) is known.

$$SV = LVdv - LVsv$$

$$CO = SV \times HR$$

It should be noted that this is the total left ventricular stroke volume; if there is significant mitral or aortic regurgitation, these parameters do not correspond to forward stroke volume or cardiac output.

B.2.4. *Mitral valve E point to septal separation (EPSS)*

This is a relatively simple parameter to measure taken from M-mode of the mitral valve. It gives an indication of global left ventricular function and left ventricular dilatation independent of ventricular geometry, size or abnormal motion (Vuille & Weyman 1994). It can be used to confirm the measured ejection fraction, and in adult humans an EPSS in excess of seven millimetres corresponds to an ejection fraction

of less than 45% (Schiller & Foster 1996). Pollick and colleagues (1982) found that EPSS was primarily a function of left ventricular size rather than fractional shortening or ejection fraction. As mitral stenosis is rare in the dog, there are few limitations to this measure in this species, although significant aortic regurgitation will affect it. Increased EPSS has high specificity but low sensitivity for DCM (Borow 1989). Kirberger (1991a) reported EPSS values ranging from 1.0 - 6.0 mm, using Beagles and German Shepherd dogs and found no significant effects of breed, weight, sex or heart rate. In children, when EPSS was normalised to the left ventricular end-diastolic diameter, there was no correlation with age, height, BSA or weight in normal subjects or patients with congenital volume overloading defects, but the EPSS: LVIDd did increase in the presence of DCM (Engle *et al* 1983). A B bump on the anterior mitral valve leaflet is indicative of impaired left ventricular systolic performance (Movsowitz *et al* 1996).

Mitral M-mode may also be used to measure early leaflet separation, between the anterior and posterior leaflets of the mitral valve, in early diastole. In DCM, both early and late (subsequent to atrial contraction) leaflet separation are reduced compared with normal individuals (Douglas *et al* 1987a). The mitral orifice area, relative to the short axis left ventricular area at mitral valve level, is reduced in DCM (Pollick *et al* 1982). This is related both to left ventricular dilatation and to reduced stroke volume, as these authors also showed that aortic orifice area, relative to aortic root diameter, was also reduced in DCM patients compared with healthy humans.

B.2.5. *Mitral annulus motion (Atrioventricular plane displacement)*

Mitral annulus motion, also known as atrioventricular plane displacement or mitral valve annulus displacement, gives an indicator of contractility in an apico-basilar direction. Circumferential fibre shortening has formed the basis for the dominant methods for ascertaining left ventricular function, such as chamber dimensions, fractional shortening and ejection fraction (Simpson 1997). However, left ventricular myocardial architecture also includes longitudinally arranged myocardial fibres, particularly in the subendocardium, within the trabeculae and the papillary muscles

(Simpson 1997). Because of a constant relation between the heart and the chest wall, atrioventricular plane displacement is a consequence of this longitudinal fibre shortening (Simpson 1997).

The events associated with and the description of mitral annulus motion have been detailed by Keren and colleagues (1988c). After about 90 milliseconds after the onset of the QRS complex, the mitral annulus is displaced towards the left ventricular apex due to contraction of the longitudinal fibres. This contraction usually occurs prior to short-axis fibre shortening, resulting in a transiently more spherical ventricle during the isovolumic contraction period (Simpson 1997). During diastole, the annulus recoils towards the atrium, corresponding to the onset of rapid ventricular filling and the onset of the D phase of pulmonary venous flow. The final displacement towards the atrium (corresponding to about 2.4 mm in normal humans) occurs after atrial systole (Keren *et al* 1988c).

The magnitude of systolic excursion in the apico-basilar dimension is measured from a four chamber and two chamber view, and the mean recorded (Alam *et al* 1990; Alam 1991; Pai *et al* 1991). Although normally measured from M-mode, it has been described from 2D techniques (Reynolds 1997). Provided four areas are assessed, the mean is a good indicator of global LV performance even in the presence of regional wall motion abnormalities (Simpson 1997). The normal mitral annulus motion in man is fifteen millimetres and measurement abnormalities are said to provide an early indication of myocardial dysfunction (Schiller & Foster 1996), particularly associated with myocardial hypoxia or ischaemia since the subendocardial longitudinal fibres are particularly susceptible to these environments (Simpson 1997). In man, mitral annulus excursion in excess of ten millimetres is likely to be associated with a normal left ventricular ejection fraction of 0.5 or 50% (Alam 1991). A mean mitral annulus motion of less than seven millimetres predicts an ejection fraction below 30%, with a sensitivity of 92% and specificity of 67% (Alam *et al* 1990).

Mitral annulus motion influences pulmonary venous flow (Keren *et al* 1988c). Magnitude of the mitral annulus motion is reduced in DCM (Keren *et al* 1988c; Alam *et al* 1990). The technique may be used to assess septal contractility even when left bundle branch block results in paradoxical septal motion (Silva *et al* 1996). The magnitude of the atrioventricular plane displacement has also been suggested to have prognostic significance (Willenheimer *et al* 1997), and has been suggested as an alternative to determination of ejection fraction, as the technique is less dependent on image quality or operator experience (Willenheimer *et al* 1997).

Schober and colleagues (1997) presented the results of mitral annulus motion in dogs, and reported a normal mean motion of 1.2 ± 0.18 centimetres for the septal annulus and showed it was decreased in dogs with mitral valve disease and was markedly low in dogs with DCM. No mention was made of the influence of body weight in this mixed breed group, although most dogs were small or medium size breeds.

B.2.6. *Systolic time intervals*

Systolic time intervals (STIs) provide a quantitative description of overall left ventricular performance reflecting the combined influences of heart disease, compensatory responses and pharmacological intervention (Henik 1995). Findings expected with diminished left ventricular performance are increased pre-ejection period (showing reduced rate of LV systolic pressure rise), reduced left ventricular ejection time (due to reduced fibre shortening), increased pre-ejection period to ejection time ratio (PEP:ET) and reduced velocity of circumferential fibre shortening (Vcf). The time for electromechanical systole (start of Q to aortic valve closure (QAVC) is normally unaltered (Henik 1995). The systolic time intervals normally fall between narrow physiologic limits and thus are highly sensitive measures of overall ventricular function (Allworth *et al* 1995). It can be argued that this is a preferable method of assessing left ventricular global function, whereas fractional shortening only assesses a small, possibly unrepresentative portion of the myocardium (Allworth *et al* 1995) and ejection fractions determined volumetrically

are prone to error. STIs are non-specific indicators of left ventricular performance because they are affected by myocardial contractility, heart rate and loading conditions, but they do correlate with invasively derived measurements of ejection fraction, cardiac output and stroke volume (Henik 1995). They can be measured from M-mode of the aortic valve from a short axis view. Although, M-mode has better temporal resolution than pulsed wave Doppler, this is not clinically significant and using Doppler aortic flow spectra has been justified (Borow 1989). Amberger and Lombard (1998) commented that precision of measurement was improved by using spectral Doppler aortic flow traces.

The pre-ejection period, PEP, is influenced by heart rate, aortic diastolic pressure, duration of intraventricular conduction, LV end-diastolic pressure and contractility (Borow 1989). In dogs, however, Pipers and others (1978) showed, using linear regression, that there was no significant relationship between PEP and heart rate, which was supported by Atkins and Snyder (1992). PEP tends to increase with left ventricular systolic failure due to decreased rate of pressure rise during isovolumic systole (dP/dt) (Borow 1989).

The ejection time (ET) is influenced by heart rate, stroke volume, preload, afterload and contractility. ET tends to shorten with systolic failure and mitral regurgitation (Borow 1989). There is a significant inverse relationship with heart rate (Pipers *et al* 1978) and it may be corrected for heart rate in various ways to give an ejection time index (LVETI). From the linear regression between HR and STI, Atkins and Snyder (1992) used the following formula in dogs:

$$LVETI = ET + (0.55 \times HR)$$

The ratio of PEP:ET is less heart rate dependant and can be used as a sensitive indicator of contractility although it does also depend on preload and afterload (Borow 1989). In dogs, there has been some controversy in the veterinary literature in the dependency of this ratio on heart rate. A clear relationship was shown by

Pipers and colleagues (1978), although the study by Atkins and Snyder (1992) and Amberger and Lombard (1998) showed no significant association. Normal values given for canine PEP:ET ratio range from 0.24 (Pipers *et al* 1978) and 0.34 (Atkins & Snyder 1992), and Amberger and Lombard (1998) suggested using a median value of 0.3 as a cut off between normal and abnormal.

The velocity of circumferential fibre shortening (Vcf) is a derivative of the systolic time interval, ET. Vcf is an indicator of the rate of contraction between systole and diastole expressed in circumferences / second although it is really a measure of the rate of change of LV size in diameters per second (Borow 1989). It is calculated from the fractional shortening (expressed as a fraction rather than a percentage) and the ejection time (Feigenbaum 1994a):

$$Vcf = FS/ET.$$

Vcf is relatively preload independent but is highly dependent on heart rate and afterload as well contractility (Borow 1989). The effect of heart rate can be minimised by correcting the value by multiplying it by the square root of the preceding R-R interval (Borow 1989). Atkins and Snyder (1992) normalised for heart rate by dividing the value of Vcf by the heart rate and multiplying by 100.

STIs have been validated in the dog using M-mode (Pipers *et al* 1978; Atkins & Snyder 1992). Amberger and Lombard (1998) showed that there should be no difference between values obtained by M-mode of the aortic valve motion and values determined from spectral Doppler of aortic flow. There are probably breed related differences in the normal values in dogs as has been demonstrated for the greyhound (Snyder *et al* 1995).

B.2.7. *Doppler indices*

B.2.7.1. *Doppler indices of aortic flow*

Doppler may be used to assess global left ventricular systolic performance by measuring parameters of aortic blood flow and results are not subject to any assumptions based on chamber size or shape (Movsowitz *et al* 1996). Peak aortic flow and the peak acceleration rate of aortic flow correlate well with global left ventricular systolic performance, although the indices are load dependent (Goldberg *et al* 1988a; Movsowitz *et al* 1996). Obviously, this can only be true in the absence of any aortic stenosis. As the best alignment with flow is usually from the subcostal view in dogs with aortic stenosis (Lehmkuhl & Bonagura 1994), it is evident that this would be the optimal view to use in determining left ventricular systolic function in the absence of aortic stenosis.

Doppler of the aortic outflow allows calculation of the forward stroke volume and the cardiac output (Goldberg *et al* 1988a;c; Borow 1989), although this requires an accurate measure of aortic diameter (any error in the radius is magnified four fold) (Feigenbaum 1994b; Movsowitz *et al* 1996). The aortic spectrum velocity time integral is a good indicator of left ventricular performance and can be used without introducing errors due to inaccuracies in measurement of vessel diameters (Feigenbaum 1994a). Aortic flow peak velocity and velocity time integral are significantly reduced in patients with DCM, with minimal overlap with data from normal healthy individuals (Gardin *et al* 1983). Similar findings are reported in dogs (Darke 1990;1992;1994; Darke *et al* 1993).

The rate of acceleration at which blood is ejected from the left ventricle (dv/dt), or the ejection force, can be used to indicate contractility, with calculation of aortic dv/dt (maximum or mean) and acceleration time (Feigenbaum 1994a). There is significant overlap between normal acceleration time and prolonged acceleration time in patients with DCM (Gardin *et al* 1983).

One confounding factor about the use of Doppler evaluation of aortic flow in DCM is the difficulty in sample volume positioning, since the velocity distribution in the aortic annulus in patients with DCM is skewed, with higher velocities obtained from the anterior and septal parts of the annulus (Zhou *et al* 1996).

B.2.7.2. *Doppler indices of mitral regurgitation*

With patients with mitral regurgitation, then a calculation which gives the gold standard indicator of contractility, dP/dt, can be used. From the acceleration slope of the spectrum, two points of known velocity are selected, allowing calculation of the pressure gradient between them (dP) from the modified Bernouille equation. The time between the two points (dt) is determined and dP/dt is calculated (Bargiggia *et al* 1989; Chen *et al* 1991; Feigenbaum 1994a; Vuille & Weyman 1994; Movoswitz *et al* 1996).

Borgarelli and others (1998) found that the velocity of mitral regurgitant jets in canine DCM was related to survival times; dogs with jet velocity less than four metres per second (m/s) had a worse prognosis than those dogs with mitral regurgitation with peak velocity exceeding four m/s.

The severity of mitral regurgitation may be subjectively or semi-quantitatively assessed by the jet area in the left atrium, assessed by pulsed wave spectral or colour flow mapping Doppler echocardiography (Helmcke *et al* 1987; Goldberg *et al* 1988b; Kisslo *et al* 1988; Perry 1989). More accurate methods of quantifying the mitral regurgitant fraction are reported, by determining the total left ventricular stroke volume (from two-dimensional echocardiographic computation, or possibly from mitral inflow pulsed wave Doppler echocardiography) and the forward stroke volume, from aortic flow velocity time integral and cross sectional area and then calculating the mitral regurgitant fraction (Blumlein *et al* 1986; Rokey *et al* 1986; Goldberg *et al* 1988c; Keren & LeJemtel 1989).

B.2.7.3. *Left ventricular meridional systolic wall stress*

This is a useful parameter to measure as it is less dependent on loading conditions compared with the other parameters of systolic function (Feigenbaum 1994a; Vuille & Weyman 1994). It is valid in the absence of aortic stenosis and is derived from M-mode indices and systolic arterial pressure (SAP), with wall stress units in dynes/cm^2.

$$\text{Stress} = \frac{0.334 \times \text{SAP} \times \text{LVIDs}}{\text{LVpws}\,[1 + (\text{LVpws}/\text{LVIDs})]}$$

Systolic arterial pressure may be measured in dogs using the Dynamap^R oscillometric method (Allworth *et al* 1995), although this significantly underestimates systolic pressure (Meurs *et al* 1996a). The Doppler technique may be more accurate (Binns, S.H. *et al* 1995).

A number of techniques have been described for calculating meridional or circumferential wall stress, relating altered geometry in cardiac disease to increasing wall stress (Marsh *et al* 1979; Quinones *et al* 1980; Douglas *et al* 1987c; Binkley *et al* 1988; Morgan *et al* 1989; Grandi *et al* 1993; Vuille & Weyman 1994; Municino *et al* 1996).

B.3. *Echocardiographic / Doppler assessment of left ventricular diastolic function*

B.3.1. *Introduction to the assessment of diastolic function*

Diastolic function is a complex sequence of many inter-related events (Nishimura & Tajik 1997) and it is therefore difficult to assess (Yamamoto *et al* 1996). For simplification, diastolic function is usually subdivided into the following different haemodynamic phases which are fundamentally different in their properties. These include isovolumic relaxation, rapid filling phase, diastasis and the late filling phase corresponding to atrial contraction (Little & Downes 1990; Taylor & Waggoner 1992; Modersohn *et al* 1993; Choong 1994). The two major determinants of ventricular filling are ventricular relaxation and effective chamber compliance (Nishimura & Tajik 1997).

Diastolic dysfunction occurs when left ventricular filling required to produce an adequate cardiac output requires an elevated pulmonary venous pressure. Systolic dysfunction is the major cause of diastolic dysfunction (Little & Cheng 1998), since an elevated end-diastolic pressure is required to maintain cardiac output, and an elevated pulmonary venous pressure may therefore be required to achieve ventricular filling. Consequently, it is not surprising that diastolic dysfunction is recognised in association with DCM. However, diastolic dysfunction is an integral part of the pathophysiology of DCM (Takenaka *et al* 1986). Failure of complete relaxation and increased myocardial stiffness may play a part in explaining the elevated filling pressures and reduced cardiac performance of DCM patients (Keren *et al* 1992). Left ventricular filling parameters are reported to correlate better with NYHA class of congestive failure than indices of systolic function in patients with DCM (Vanoverschelde *et al* 1990).

B.3.2.1. *Mitral inflow parameters in the assessment of diastolic function*

For over a decade, it has been appreciated that study of mitral inflow patterns provide information about left ventricular diastolic function (Labovitz & Pearson 1987; Harizi *et al* 1988; Little & Downes 1990; Taylor & Waggoner 1992). Doppler echocardiography is reported to compare favourably with radionuclide angiographic techniques (Spirito *et al* 1986; Pearson *et al* 1988) and gated blood pool scintigraphy (Friedman *et al* 1986) in the evaluation of left ventricular diastolic function. Although various cardiac diseases are associated with abnormal mitral inflow patterns, it should be noted that these patterns are related more to myocardial function and haemodynamics than the specific type of disease process (Appleton *et al* 1988).

During early diastole, and the isovolumic relaxation period, myocardial relaxation results in a steep exponential fall in intraventricular pressure as the elastic components of the left ventricle, compressed and twisted during ejection, are allowed to recoil. These processes allow rapid filling after opening of the mitral valve. The

left ventricular pressure in the normal heart should continue to decline despite the increase in volume associated with filling (it is the left atrial to left ventricular pressure gradient which is responsible for the rapid filling phase) (Little & Cheng 1998). This represents the E peak of mitral inflow. Left atrial pressure is the most important determinant of the velocity and shape of the E wave (Lewis 1996) although left ventricular function influences it (Nakatani *et al* 1992). Factors listed by Yamamoto and colleagues (1996) as influencing the mitral E wave are the atrial factors (left atrial pressure and compliance) and the ventricular factors (left ventricular relaxation, elastic recoil, compliance and interventricular interaction).

During diastasis, small late (L) waves may be evident. The other major phase of left ventricular filling is the A wave subsequent to atrial contraction. The A wave reflects the contractile state of the left atrium, the left atrial: left ventricular instantaneous pressure gradient and the state of left ventricular compliance (Lewis 1996). Normally, most flow is early in diastole so E peaks are higher velocity and have larger velocity time integral than A peaks (Labovitz & Pearson 1987; Harizi *et al* 1988; Spirito & Maron 1988b; Smith 1990). In patients with fast heart rates (especially cats) E and A peaks may be poorly separated or even summated (Frey & Douglas 1990; Santilli & Bussadori 1998). In man, increasing age reduces the E:A ratio, but the parameters of mitral inflow are not influenced by body size (Spirito & Maron 1988b). Other parameters assessed in diastolic function is the E wave deceleration time (Klein & Cohen 1996) or the E wave deceleration half-time (Takenaka *et al* 1986). In normal individuals, left atrial diameter, left ventricular wall thickness or chamber diameter, left ventricular mass and patient body mass index are reported to have little or no association with Doppler indices (Benjamin *et al* 1992). However, in the presence of left ventricular hypertrophy or left ventricular dilatation, the E to A ratio and the A velocity time integral as a fraction of total inflow does correlate with the left ventricular radius: wall thickness ratio, over-riding the effects of age (Sartori *et al* 1987). Mitral A wave and A:E ratio is related to an interaction of left ventricular chamber volume and mass and not just chamber size or hypertrophy alone (Kenny *et al* 1991).

Sample volume position also influences mitral inflow pattern (Feigenbaum 1994a; Choong 1994) and positioning between mitral valve leaflet tips is normally advocated (Klein & Cohen 1996; Appleton *et al* 1997), which optimises E:A ratio (Choong 1994; Mantero *et al* 1998). Other authors, however, suggest positioning at the mitral annulus (Frey & Douglas 1990). The image plane is also important. Mitral inflow velocities are approximately four percent higher if obtained from the apical four chamber view compared with the apical two chamber view (Gardin *et al* 1986).

Abnormal LV diastolic function due to impaired relaxation may be recognised by:

- E:A < 1 (i.e. A>E)
- prolonged isovolumic relaxation time (IVRT)
- Prolonged deceleration time

These features characterise impaired relaxation. The fall in diastolic left ventricular pressure is reduced, compromising the early filling phase, so LV filling is more dependent on atrial contraction (Klein & Cohen 1996; Masuyama & Popp 1997).

If the underlying condition evolves to result in more severe impairment of diastolic function, and left atrial pressure increases, this compensates for the impaired relaxation pattern, since there is a more rapid rate of early diastolic transmitral flow and a faster deceleration time (Little & Cheng 1998). Consequently, superficial scrutiny of the mitral inflow pattern may not detect the presence of diastolic dysfunction in the patient under examination; the transmitral pattern is described as "pseudonormalised" (Nishimura & Tajik 1997). In man, Valsalva manoeuvres may unmask a pseudonormal pattern (Masuyama & Popp 1997). Mitral regurgitation may also play a role in pseudonormalisation (Werner *et al* 1994).

In the presence of even more severe diastolic function with a markedly elevated left ventricular pressure, this more than offsets the impaired relaxation pattern, and a

restrictive transmitral flow pattern may be recognised (Masuyama & Popp 1997; Little & Cheng 1998):

- E:A > 2
- decreased IVRT
- decreased deceleration time

The increase in left ventricular stiffness results in brief early flow duration, decreased E wave deceleration time and increased deceleration rate. These features characterise a restrictive left ventricle (Klein & Cohen 1996). During the rapid filling phase, the stiff left ventricle abruptly restricts further filling, and atrial contraction offers minimal additional filling. These changes can also be seen with elevated filling pressures associated with congestive heart failure, significant mitral regurgitation and left atrial and left ventricular volume overload, reduced compliance of the left ventricle or restrictive myocardial disease (Klein & Cohen 1996). With increased left ventricular volumes, pericardial restraint becomes significant and shifts the diastolic pressure-volume curve upwards and leftwards, which explains the increased E wave velocity and decreased deceleration time identified with increasing preload (Nishimura & Tajik 1997). The fact that these different mitral inflow patterns evolve with disease progression is not actually proven, but is a hypothesis supported by most authors (Cohen *et al* 1996; Appleton *et al* 1988; Nishimura & Tajik 1997; Oh *et al* 1997) and has been demonstrated in cardiac amyloidosis (Abdulla *et al* 1998).

Mitral inflow patterns are also affected by changes in LV volume (Fragata & Areias 1996). Decreased preload (Castini *et al* 1992), nitroglycerine administration (Choong *et al* 1987) or ACE inhibitor therapy (Keren *et al* 1992) may mimic diastolic dysfunction. In contrast, increased preload may result in mitral inflow patterns masking diastolic dysfunction (Stoddard *et al* 1989), as may the presence of mitral regurgitation (Takenaka *et al* 1986), although this effect depends on the presence of elevated filling pressures (Lavine & Arends 1989). Changes in posture and exertion may also influence transmitral flow (Kmetzo *et al* 1991). Caution was urged in interpreting diastolic abnormalities from trans-mitral filling patterns, as the

conclusions may be diametrically opposed to actual haemodynamic changes, as shown in a study where catheterisation data indicated improved diastolic function with an amrinone infusion, whereas mitral inflow patterns suggested reduced LV compliance (David *et al* 1989). Other determinants of Doppler indices of diastolic function are the P-R interval, left ventricular systolic function and systolic blood pressure (Benjamin *et al* 1992) and gender (Kangro *et al* 1996).

The fact that normal mitral flow patterns will vary with loading conditions, age and heart rate confounds the interpretation of patterns identified in various cardiac diseases (Nishimura *et al* 1989; Nishimura & Tajik 1997). Despite these confounding factors, a grading system for the severity of diastolic dysfunction has been proposed by Nishimura and Tajik (1997). A grade I pattern corresponds to abnormal relaxation, grade II to pseudonormalisation, grade III to a restrictive filling pattern which improves with treatment and grade IV represents a restrictive filling pattern which is maintained despite therapy, indicating severe abnormalities of left ventricular compliance and end-stage heart disease. Peak E velocity and short E deceleration time were the strongest negative predictors of survival in patients with DCM, from a Cox proportional hazards analysis (Werner *et al* 1994). A number of authors support the fact that unresolving mitral inflow restrictive patterns are associated with poor prognosis (Giannuzzi *et al* 1996; Nishimura & Appleton 1996; Traversi *et al* 1996; Xie *et al* 1996; Lee *et al* 1997; Pinamonti *et al* 1997).

Tenenbaum and colleagues (1996) showed that measurement of the A wave deceleration time was also informative; if shortened, it indicated elevated left ventricular filling pressure. Castini and others (1992) reported that the peak A velocity and A velocity time integral were independent of preload reduction (unlike the E wave) and could provide an index of diastolic function. A positive relationship between the A wave velocity and the body mass index suggests that left ventricular compliance is reduced in obese individuals (Lewis 1996). Other factors influencing the A wave have been listed (Yamamoto *et al* 1996).

The ratio of velocity time integrals less than one (Evti:Avti < 1) is insensitive but specific at detecting abnormal left ventricular compliance. Calculation of the atrial contribution to total left ventricular filling (Avti/(Evti+Avti) may also indicate impaired relaxation when increased as long as left atrial function is normal (Choong 1994).

Scalia and colleagues (1997) reported on a noninvasive technique for estimating the ventricular relaxation time constant, *tau* (τ), from Doppler derived IVRT, peak systolic blood pressure and left atrial pressure (determined from the pulmonary capillary wedge pressure on right sided catheterisation or presumed at 10 mmHg). τ was underestimated but correlated well with the invasively determined method.

It has only been recently that diastolic dysfunction has been appreciated to be a factor in DCM. Ng and Gibson (1991) described "E fillers" and "A fillers" depending on the mitral inflow pattern shown. DCM patients commonly show altered diastolic filling parameters (Fruhwald *et al* 1997b) which are of prognostic significance (Werner *et al* 1994). Left ventricular diastolic function is an integral component of end-stage DCM (St.Goar *et al* 1991). It is interesting to note that in doxorubicin-induced cardiomyopathy, left ventricular diastolic abnormalities precede systolic dysfunction (Marchandise *et al* 1989). Normally in DCM, E and A velocities are reduced and E:A ratio is increased (Sadaniantz *et al* 1997), but mitral regurgitation has the effect of increasing E masking these findings. Peak E velocity and E:A ratio correlate with the severity of mitral regurgitation, although the E deceleration time is not affected by mitral regurgitation (Thomas *et al* 1998).

The deceleration time of the early mitral inflow wave is determined by the effective stiffness of the left ventricle. In man, if it is less than 125 - 150 milliseconds, this is associated with a poor prognosis and if less than 115 - 130 milliseconds, this is associated with a grave prognosis (Pinamonti *et al* 1993; Little & Cheng 1998) regardless of rhythm (Hurrell *et al* 1998). In man, short deceleration time is more sensitive than low ejection fraction in the prediction of adverse prognosis (Pinamonti

et al 1993; Rihal *et al* 1994). The peak E velocity and E:A ratio also offers independent prognostic information (Werner *et al* 1994; Lapu-Bula *et al* 1998). Borgarelli and colleagues (1998) also showed a trend to decreased survival time in dogs with a restrictive mitral inflow pattern, although this was not statistically significant.

The informativeness of Doppler indices of diastolic function have been hampered by difficulties in obtaining accurate and reproducible measurements. Appleton and colleagues (1997) indicated some of the technical pitfalls which may be responsible for poor reproducibility. One factor was proper alignment with mitral inflow, which must take into account the size and geometry of the left ventricle. In the normal human heart, mitral inflow direction is approximately 20^{o} to the apex. In a dilated heart, mitral inflow is orientated more laterally and may be 30 - 40^{o} to the apex, and so transducer position must be modified from the standard apical view. The mitral E wave deceleration time is most affected by technical errors of alignment and sample volume placement (Appleton *et al* 1997), which is unfortunate as this parameter is of critical importance in obtaining prognostic information in DCM (Giannuzzi *et al* 1996).

The peak filling rate of the left ventricle has been demonstrated to be a useful index for evaluating diastolic function and to monitor the effect of therapeutic interventions. It has conventionally been normalised to end-diastolic volume, during radionuclide studies. Bowman and colleagues (1988) proposed that if the peak filling rate was instead normalised to the mitral stroke volume, this provided an index relatively free from geometrical assumptions, diameter measurements and errors in sample volume positioning. The peak filling rate (PFR) (expressed as stroke volumes per second; (SV/s)) is given by:

PFR = E wave velocity / total mitral velocity time integral

Excellent correlation was shown with radionuclide methods. Bowman and colleagues (1988) postulated that this index would provide a more reliable and reproducible method for assessing left ventricular filling. Santilli and Bussadori (1998) described its use in the cat as a method of correcting for errors of transducer orientation or sample volume positioning, to allow comparisons between individuals to be made.

B.3.2.2. *Intraventricular transmission of mitral flow*

Colour flow M-mode of mitral inflow from a left apical view has also been used to assess diastolic function. The propagation velocity is defined as the slope of the wave front during early filling, and it correlates well with spectral Doppler indices of mitral inflow (Brun *et al* 1992; Cohen *et al* 1996). The velocity is related to peak E velocity and varies inversely with IVRT. In patients with diastolic dysfunction, the velocity of flow propagation is reduced and Brun and colleagues (1992) proposed that this would be an important tool in investigating diastolic function. It is an ideal technique because of the high sampling rate offering the ability to measure flow velocities in temporal and spatial domains (Nishimura & Appleton 1996). Mego and colleagues (1998) described this technique in the assessment of left ventricular filling characteristics and showed that age resulted in decreased propagation velocity and the technique compared favourably with other mitral inflow Doppler indices in healthy individuals. In a pacing model of DCM dogs, colour M-mode of mitral inflow was found to be more sensitive than the conventional Doppler indices in the detection of early diastolic function during evolution of the disease (Crabbe *et al* 1997). Propagation velocity is slowed in human patients with acute myocardial infarction (Steine *et al* 1998). Flow propagation is slow in myocardial disease associated with a restrictive physiology, but is increased with constriction (e.g. pericardial disease). This slope is decreased as *tau* (τ), -dP/dt and minimal early diastolic pressure increase (Cohen *et al* 1996). Takatsuji and co-workers (1996) described a modification of this technique.

Spectral Doppler studies of mitral inflow mapped from mitral valve leaflets towards the left ventricular apex have also been assessed in various ways (Jacobs *et al* 1990;

Sniderman *et al* 1997; Kozan *et al* 1998). Velocity declines and propagation is slowed with diastolic dysfunction. The best estimate of the pulmonary capillary wedge pressure was reported to be the ratio of the mitral E wave and the flow propagation velocity (Garcia *et al* 1997).

In the normal left ventricle, there is normally rapid reversal of mitral inflow towards the left ventricular outflow tract. Pennestri and others (1992) showed that abnormal persistence of systolic flow towards the left ventricular apex on colour flow M-mode of mitral inflow was evident in both regional and global left ventricular dysfunction, which could be measured by a flow persistence index.

Fujimoto and others (1995) noted that in DCM, there is narrowed effective mitral orifice area. This, as well as the increased left atrial to left ventricular pressure gradient, may explain the increased inflow accelerations. In normal individuals, the mitral inflow signal disappears by three or four centimetres within the left ventricle but may persist up to seven or eight centimetres in DCM patients (Fujimoto *et al* 1995), suggesting that mitral inflow may be regarded as a column of blood, longer and thinner in DCM patients. Similar findings were reported by Yamamoto and colleagues (1995), who demonstrated that these changes could unmask pseudonormalised mitral inflow and were also present in patients with atrial fibrillation.

Pai and Stoletniy (1997; 1998) further examined the fate of the mitral inflow wave within the left ventricle and postulated that study of the intra-ventricular transmission of the mitral inflow E wave would provide information about the global left ventricular diastolic performance. The early mitral E wave is transmitted along the postero-lateral wall towards the left ventricular apex, where it is reflected towards the left ventricular outflow tract, and may be identified as an Er wave. The E-Er interval was prolonged in patients with abnormal relaxation (Pai & Stoletniy 1998). The mitral A wave is also reflected in a similar manner as an Ar wave (Pai & Stoletniy 1997). Brennan and colleagues (1997) reported that the A-Ar interval was an index of

left ventricular diastolic function reflecting its intrinsic elasticity and end diastolic pressure.

B.3.2.3. *Effect of age*

Age related changes result in a decreased rate of early filling, due to a reduced left atrial to left ventricular pressure gradient as a consequence of slowed left ventricular relaxation (Little & Cheng 1998). This results in a lower E to A wave ratio associated with age. Age related changes affecting mitral inflow are decreased E, increased A, increased A velocity time integral, decreased E to A velocity and velocity time integral ratios and increased E deceleration time (Störk *et al* 1990/91; Mantero *et al* 1995). Mitral E wave duration also increases in healthy elderly people, associated with decreased left ventricular compliance (Manyari *et al* 1985). In healthy humans over seventy years of age, an inverted E to A ratio is normal (Benjamin *et al* 1992; Mantero *et al* 1995). Age is the major independent variable influencing diastolic function parameters, with a greater influence than heart rate (Mantero *et al* 1998). These changes probably reflect changing collagen content, cardiomyocyte atrophy and the effect of disease (Lewis 1996). Benjamin and others (1992) reported that the mechanisms of change in diastolic function associated with age remained unexplained. They postulated that concurrent age-related disease such as myocardial ischaemia, hypertension or diabetes mellitus may be associated with these changes. An intricate combination of cellular hyperplasia, cell death, fibrosis, reduced calcium sequestration and increased passive stiffness may underlie the changes in diastolic function with ageing (Benjamin *et al* 1992). Manyari and others (1985) considered that age related changes could be considered as intracellullar (increase myofibre vacuolisation) or extracellular (increased collagen, fibrous and adipose tissue and calcium and amyloid deposition). The incidence of diastolic dysfunction is age related and heart failure due to diastolic dysfunction is markedly increased in elderly humans (Nishimura & Tajik 1997). Yu and Sanderson (1997) showed that age and heart rate exerted similar although weaker effects on mitral inflow parameters of diastolic function in patients with congestive heart failure. A weak relationship between age and the atrial filling fraction is maintained in the presence of left

ventricular hypertrophy. In DCM, the relationship between the Doppler flow indices and age is lost (Kuo *et al* 1987). In general, mitral inflow patterns are significantly affected by age and comparison should be made with published reference ranges based on gender, age and heart rate (Benjamin *et al* 1992; Mantero *et al* 1995; Nishimura & Tajik 1997). Such information is lacking in the veterinary literature.

B.3.3. *Isovolumic relaxation time*

Ventricular relaxation begins in mid-systole and continues through the first third of diastole (Nishimura & Tajik 1997). If this relaxation is slow or incomplete, left ventricular distensibility is reduced (Little & Cheng 1998). IVRT depends on this myocardial relaxation as well as the pressure gradients between the left atrium and left ventricle and the left ventricle and the aorta (Little & Cheng 1998). IVRT is significantly correlated with the acceleration of the E wave of mitral inflow and the IVRT and the A:E ratio show a linear relationship (Brecker *et al* 1992). IVRT increases with age (Spirito & Maron 1988a).

IVRT may be measured using pulsed wave Doppler with the sample volume positioned in the left ventricular outflow tract so detecting left ventricular outflow and mitral inflow (Borow 1989). Lee and others (1990) found that the IVRT was 25 $\pm$ 10 milliseconds longer if measured by Doppler compared with the mitral M-mode technique, unless phonocardiography to identify S2 was used, showing that the two measurement techniques are not interchangeable. The IVRT increased with volume loading (Fragata & Areias 1996).

B.3.4. *Pulmonary venous flow*

Although the right ventricle generates the energy driving pulmonary venous flow (PVF), the actual pattern of PVF is determined by haemodynamic events in the left atrium which in turn are influenced by left ventricular events (Choong 1994). The pulmonary veins are thin walled, very compliant structures which may accommodate a potential reservoir of more than one stroke volume of blood (Klein & Tajik 1991). PVF has been suggested as a noninvasive semiquantitative method of assessing left

ventricular filling pressure (Steen *et al* 1994). There are three major phases to PVF (Klein & Tajik 1991; Feigenbaum 1994a; Weyman 1994a; Choong 1994). After the P wave on the ECG, a small wave passes retrogradely into the pulmonary veins associated with atrial systole (Ar or R). During subsequent atrial relaxation, forward flow in the pulmonary veins may be seen, called the early systolic flow (S_E) associated with resulting fall in left atrial pressure. Associated with ventricular systole, and the movement of the mitral annulus apically, the major forward systolic flow is identified in the pulmonary veins (S_L) resulting from the increased left atrial volume and fall in left atrial pressure. It has been proposed that there is a direct linear relationship between the velocity of peak S and cardiac output (Weyman 1994a). The S_L waves is also influenced by right ventricular stroke volume (Appleton *et al* 1997). These two phases may not be distinctly separate and may be represented as a single systolic phase (S). During ventricular diastole, the left atrium functions as a conduit between the pulmonary veins and the left ventricle and during this time, in normal animals, the major forward flow (D) can be recognised in the pulmonary veins. D corresponds to the L (diastasis) phase of mitral inflow (Choong 1994) and peak D is about 50 milliseconds behind peak E (Weyman 1994a). Normally, the D wave is of higher peak velocity than the S wave. There is a small reversal wave (R_2) recognised in dogs and other species by Schober and colleagues (1995; 1996; 1998) demarcating the S and D phases, which probably represents recoil of the mitral annulus. Although this feature was evident in figures included in publications about human PVF, it was not noted until Klein and Cohen (1996) called it a late systolic reversal wave (Vr), who commented it resembled an equivalent but more pronounced and better recognised late systolic reversal wave in systemic venous flow.

Study of PVF gives further insights into diastolic function in an individual (Oh *et al* 1997). Klein and Tajik (1991) and Yamamoto and others (1996) listed the factors which were important in determining the various PVF wave forms.

Spontaneous respiration has little effect on the PVF pattern (Klein *et al* 1998). The S:D ratio tends to increase with faster heart rates, because of reduced diastolic filling

time (Klein & Tajik 1991; Steen *et al* 1994). With advancing age, the S wave has higher and the D wave lower velocities, so increasing the Sv:Dv ratio (Klein & Tajik 1991). Age is said to be principle determinant of PVF parameters (Gentile *et al* 1997).

Although it is not possible to determine left atrial pressure from the PVF, there is strong negative correlation between LA pressure and the systolic forward velocity as a percentage of overall forward velocity i.e. Sv/(Sv+Dv) x 100% (Klein *et al* 1997; Choong 1994) and the systolic fraction of total forward flow (Svti/total forward vti) (Klein & Tajik 1991). A systolic fraction less than 40% is predictive of increased mean left atrial pressure (Klein *et al* 1998). The ratio of PVF Ar and PVF S_E waves are also reported to correlate positively with mean pulmonary capillary wedge pressure (PCWP) (Tabata *et al* 1997). Multiple linear regression equations have been reported as significantly predicting a relationship between PCWP, validated by right heart catheterisation. These involve a positive association between mitral E deceleration rate (peak velocity divided by deceleration time) with a negative association of the systolic fraction of forward PVF (Pozzoli *et al* 1996). The equations are predictive even in the presence of mitral regurgitation.

If there is impaired left ventricular relaxation, the D wave is reduced and the Ar wave increases (Feigenbaum 1994a), as the abnormal left ventricle receives reduced amounts of blood from the left atrium and atrial pressures prior to atrial contraction are higher than normal, so atrial systole is associated with increased back flow into the pulmonary veins as well as into the left ventricle. Increased left ventricular end-diastolic stiffness is associated with an increased Ar wave. With elevated LV filling pressures, mitral A velocity is diminished as Ar increases (Ito *et al* 1998). In man, the velocity exceeds 35 centimetres per second in the presence of a restrictive or a pseudonormalised mitral inflow pattern, unless there is atrial systolic dysfunction or atrial fibrillation (Little & Cheng 1998). In the presence of mitral inflow pseudonormalisation, PVF D wave velocity greater than 60 cm/s or an Sv:Dv ratio of less than 0.75 will unmask this finding, although will only identify about sixty

percent of patients with the pseudonormal transmitral filling pattern (Masuyama & Popp 1997). The ratio of PVF Ar to mitral A wave velocities (Arv:Av) is strongly correlated with the mean PCWP. A ratio exceeding 0.5 predicts a PCWP greater than 15 mmHg with 88% sensitivity and 80% specificity (Ito *et al* 1998) and consequently is a useful marker for elevated PCWP in patients with systolic dysfunction.

The difference in durations between the mitral A wave and the PVF Ar wave is also informative. PVF Ar duration exceeds A duration in patients with a left ventricular end-diastolic pressure (LVEDP) of greater than 15 mmHg (Yamamoto *et al* 1997; Abdalla *et al* 1998; Klein *et al* 1998). The differences in durations are relatively independent of age in normal human subjects (Klein *et al* 1998). The magnitude in the difference between the two durations reflects the severity of the diastolic dysfunction. Differences increase in diastolic dysfunction both by prolongation of Ar and shortening of mitral A duration (Abdalla *et al* 1998).

PVF corresponding to the restrictive pattern of mitral inflow may be recognised as a small S and a large D, with large atrial wave (Klein & Tajik 1991; Feigenbaum 1994a). During early passive filling, rapid ventricular filling is mirrored by rapid pulmonary venous diastolic forward flow. As left atrial pressures are increased, mitral annulus motion does not significantly increase left atrial volume so less systolic forward flow is recognised. Atrial contraction into a stiff left ventricle means that more blood follows the favourable pressure gradient retrogradely into pulmonary veins giving large Ar.

In patients with atrial fibrillation, 34% were reported to only have a D wave although the remainder showed an S_L and a D wave (Chirillo *et al* 1997). In this study, if the D wave duration and D wave deceleration time was normalised to the heart rate (by dividing by the square root of the R-R interval), a D wave deceleration time of more than 220 milliseconds predicted a mean PCWP exceeding 12 mmHg with 100% sensitivity and 100% specificity (Chirillo *et al* 1997).

Mitral regurgitation, by influencing pressure changes within the left atrium, also exerts an effect on PVF patterns. The S wave may be blunted or even reversed in patients with severe MR (Weyman 1994a; Pieper *et al* 1996; Seiler *et al* 1998). The S:D velocity time integral ratio may be used to semi-quantitatively classify the severity of mitral regurgitation (Klein *et al* 1997; Seiler *et al* 1998).

In human patients with DCM, the S wave may be reduced or even absent due to poor mitral annulus motion, mitral regurgitation or the presence of atrial fibrillation (Klein & Tajik 1991; Klein *et al* 1997; Kranidis *et al* 1994) and associated diastolic dysfunction may fail to augment the Ar wave; this is probably due to intrinsic left atrial dysfunction (Ito *et al* 1996). A PVF S:D velocity ratio <0.7 in man indicates severely compromised LV function with elevated left atrial pressure (Kranidis *et al* 1994).

Canine reference values for PVF including data from fourteen normal dogs have been reported (Schober *et al* 1998). Some abnormalities in dogs with various heart diseases have also been illustrated (Schober *et al* 1995; 1996).

Technical problems may confound the use of PVF wave forms in the assessment of diastolic function, and Appleton and colleagues (1997) gave a useful technical guide in optimising PVF signals and reproducibility. In man, it is recommended that the right upper pulmonary vein is interrogated, and it is this, or the right middle pulmonary vein, recommended in dogs (Schober *et al* 1998).

B.4. *Combined myocardial performance (systolic and diastolic function)*

Tei and colleagues (1995; 1996; 1997) proposed a global index of myocardial performance (IMP) combining systolic and diastolic function. This is equivalent to the sum of isovolumic contraction time and isovolumic relaxation time, divided by the left ventricular ejection time. The sum of the isovolumic indices are determined by measuring the duration between cessation of mitral inflow during one cardiac cycle and onset of mitral inflow in the subsequent cardiac cycle. The ejection time is subtracted from this duration to give the sum of the isovolumic contraction and relaxation times. In patients with severe DCM, this index was much greater than in normal individuals, with much less inter-group overlap compared with other parameters (Tei *et al* 1995). In the assessment of biopsy-proven amyloidosis, the isovolumic times increased and the ejection time decreased, resulting in a marked increase in the index (Tei *et al* 1996). In normal humans, the index is 0.39 ± 0.04, compared with amyloidosis patients, with a mean index result of 0.81 ± 0.21. The index, ejection time and mitral E deceleration time were all significant predictors of outcome (Tei *et al* 1996). The index is reported to correlate well with invasive measurements of systolic and diastolic function (Tei *et al* 1997). It has been well received by human cardiologists (Burgess 1998).

B.5. *Echocardiography / Doppler: Canine reference values*

Reference values for normality are required prior to detecting abnormalities indicative of chamber dilatation or systolic or diastolic dysfunction. Although echocardiography has become the gold standard in the diagnosis of DCM (Keene 1989; ISACHC 1995), it is important to understand the limitations of echocardiography (Sisson & Thomas 1995).

Since the advent of M-mode echocardiography in veterinary cardiology (Mashiro *et al* 1976; Dennis *et al* 1978; Bonagura 1983), knowledge and application of ultrasound techniques has exploded, although until recently, publications giving reference values for M-mode parameters have been based almost solely on weight (Mashiro *et al* 1976; Boon *et al* 1983; Lombard 1984b; Bonagura *et al* 1985; Jacobs & Mahjoob 1988b; Miller *et al* 1989; Lusk & Ettinger 1990) and many of these publications cite the work of previous authors for their reference values. These reference ranges were generated for various breeds or mongrels of different weights, up to only about 35 kilograms. The failure to take into account the possible effects of breed may explain in part the wide confidence intervals for the regression lines published (Boon *et al* 1983; Lombard 1984b; Bonagura *et al* 1985; Jacobs & Mahjoob 1988b; Miller et al 1989, Lusk & Ettinger 1990; DeMadron 1996). Another confusing factor in the literature is the fact that most authors publish the regression lines for each parameter, with 95% confidence intervals for the line (e.g. Bonagura *et al* 1985), but do not show 95% prediction intervals or the actual data points, although Lombard (1984b) does show both confidence intervals and prediction intervals and Jacobs and Mahjoob (1988a;b) use prediction intervals although confusingly called them confidence intervals of predicted values.

Expected echocardiographic values based on body weight alone are most likely simplistic and presumptive because of the wide variation in canine size and somatotypic conformation (Morrison *et al* 1992). Echocardiographic and M-mode parameters will vary with breed. As more data becomes available for dogs, breed must be considered in establishing echocardiographic measurement reference ranges

(Henik 1995) and some breed-specific M-mode values have been published for Dobermanns (Calvert *et al* 1982; Calvert & Brown 1986), cocker spaniels (Gooding *et al* 1986a), beagles (Crippa *et al* 1992), Pembroke corgis, miniature poodles, Afghan hounds and golden retrievers (Morrison *et al* 1992), Boxers (Herrtage 1994), English setters (Pietra *et al* 1998), Irish wolfhounds (Vollmar 1996), great Danes (Borgarelli *et al* 1996) and non-racing greyhounds (Snyder *et al* 1995) and greyhounds undertaking training (Lonsdale *et al* 1998). Most authors use only three measurements of each parameter.

To minimise the effect of body weight or BSA, a number of authors have presented regression lines to take into account this variable when it affects parameters (Boon *et al* 1983; Lombard 1984b; Morrison *et al* 1992; Freeman *et al* 1996). Jacobs and Mahjoob (1988b) used multiple regression analysis with a derivative of heart rate, the square root of the preceding R-R interval as well as body weight to attempt to reduce confidence intervals further. The fact that specific breeds also differ in specific ways from published regression equations has only recently been appreciated. Borgarelli and colleagues (1996) reported that great Danes had much smaller left ventricular dimensions than would be predicted by published regression equations. Snyder and co-workers (1995) superimposed greyhound echocardiographic parameters on published regression lines to demonstrate the differences for this breed. The fact that regression lines with different slopes and different intercepts for specific breeds suggest that lines generated from a mixture of breeds should not be used to elucidate reference ranges of M-mode parameters for specific breeds (Morrison *et al* 1992).

It is apparent that reference echocardiographic values depend not just on body weight or BSA, but breed, gender (even after correcting for mass or BSA) and work (Lonsdale *et al* 1998). Echocardiographic parameters are also affected by growth. Sequential echocardiographic examinations of growing pups show that cardiac measurements increase in a curvilinear fashion relative to body weight (Henik 1995).

Some echocardiographic reference values cited in the literature, largely derived from M-mode, are displayed in Table B.1. References are given. Despite the advances in other areas of echocardiography, M-mode measurements still remain the method of choice in determining chamber size and wall dimensions (Luis Fuentes & Bonagura 1998).

Normal values for systolic time intervals based on the aortic M-mode have been published (Pipers *et al* 1978; Atkins & Snyder 1992; Amberger & Lombard 1998) but these may vary with breed (Snyder *et al* 1995). O'Grady and colleagues (1986) used two-dimensional echocardiography to generate some reference values of a number of parameters by this method for eighteen dogs of various breeds. They included regression equations for data significantly correlated to body weight. They also reported on repeatability studies and intra-observer variability studies.

Table B.1.
M-mode echocardiography reference values for dogs from the veterinary literature

Author	Breed	RVd (mm)	IVSd(mm)	IVSs (mm)	LVpwd (mm)	LVpws (mm)	LVIDd (mm)	LVIDs (mm)
Gooding *et al* 1986a	Cocker spaniel	x	0.82 (0.13)	x	0.79 (0.11)	x	3.38 (0.33)	2.22 (0.28)
Calvert *et al* 1982	Dobermann	x	10.5 (3.77)	16.5 (1.18)	10.8 (0.987)	16.2 (0.753)	49.7 (5.61)	33.8 (3.601)
Calvert & Brown 1986	Dobermann	x	9.61 (1.19)	14.3 (0.65)	9.59 (0.6)	14.1 (0.84)	46.8 (4.16)	30.8 (3.31)
Calvert 1995a	Dobermann	x	>7	>10	>7	>10	<50	x
Crippa *et al* 1992	Beagle	x	6.7 (1.1)	9.6 (1.5)	8.2 (1.9)	11.4 (1.9)	26.3 (3.4)	15.7 (3.4)
Morrison *et al* 1992	Pembroke corgi	10*	8*	12*	8*	12*	32*	19*
Morrison *et al* 1992	Miniature poodle	4*	5*	8*	5*	8*	20*	10*
Morrison *et al* 1992	Afghan hound	10*	10*	13*	9*	12*	42*	28*
Morrison *et al* 1992	Golden retriever	13*	10*	14*	10*	15*	45*	27*
Herrtage 1994	Boxers	x	9(2)	13(2)	10(2)	15(2)	40(5)	x
Snyder *et al* 1995	Greyhound	x	13.4(1.72)	x	11.6(1.67)	x	46.9(3.05)	33.3(2.61)
Pietra *et al* 1998	English Setter	x	8.1(1.5)	13.2(1.5)	7.1(1.7)	11.8(2.7)	39.7(6.1)	24.3(5.7)
Vollmar 1996	Irish Wolfhound	26.7 (5.3)	8.0 (1.9)	13.7 (3.4)	9.5 (2.1)	15 (3.3)	53.9 (5.2)	35.2 (4.8)
Brownlie, unpublished 1995	Irish Wolfhound - males	x	10.5(2.23)	12.73(2.11)	10.19(1.67)	14.96(1.91)	51.88(5.67)	37.81(4.17)
Brownlie, unpublished 1995	Irish Wolfhound - females	x	10.14(2.2)	12.29(2.21)	9.75(1.56)	14.08(2.03)	49.00(6.79)	35.75(5.64)
Bodey 1998	Scottish Deerhounds	x	10.2	12.4	10.4	13.3	47.9	36.5
Borgarelli *et al* 1996	Great Dane	x	x	x	x	x	x	x
Tidholm & Jonsson 1996	Newfoundland	x	x	x	x	x	3.5 - 6.0	2.2 - 4.4
Koch *et al* 1996	Newfoundland Irish wolfhound Great Dane	19* (6-28) 17* (10-26) 19* (11-26)	11.5* (7-15) 12* (09-14.5) 14..5* (12-16)	15* (11-20) 15* (11-17) 16.5* (14-19)	10* (8-13) 10* (9-13) 12.5* (10-16)	15* (11-16) 14* (11-17) 16* (11-19)	50* (44-60) 50* (46-59) 53* (44-59)	35.5* (29-44) 36* (33-45) 39.5* (34-45)
Mashiro *et al* 1976	Mongrels	x	✓	x	✓	x	✓	✓
Boon *et al* 1983	Various	x	✓	✓	✓	✓	✓	✓
Lombard 1984b	Various	✓	✓	x	✓	x	✓	✓
Bonagura *et al* 1985	Unspecified	x	✓	✓	✓	✓	✓	✓
Jacobs & Mahjoob 1988b	Mongrels	x	✓	✓	✓	✓	✓	✓
Lusk & Ettinger 1990	Unspecified	✓	✓	✓	✓	✓	✓	✓
DeMadron 1996	Various	✓	✓	x	✓	x	✓	✓
Pipers *et al* 1978	Various	x	x	x	x	x	x	x
Atkins & Snyder 1992	Various	x	x	x	x	x	x	x

Abbreviations As in text. ✓ = reference value given x = reference value not given. Specific values, where reported, are shown as mean (standard deviation). * = median given instead.

Table B.1.
M-mode echocardiography reference values
for dogs from the veterinary literature..... continued / 1

Author	Breed	FS (%)	EF (%)	LA (mm)	Ao (mm)	PEP (secs)	ET (secs)	PEP:ETratio	Vcf (circs/s)
Gooding *et al* 1986a	Cocker spaniel	34.26 (4.54)	x	x	x	x	x	x	x
Calvert *et al* 1982	Dobermann	31.8 (3.81)	x	x	x	x	x	x	x
Calvert & Brown 1986	Dobermann	34.2(1.81)	x	26.63(1.5)	29.9(2.31)	0.07(0.01)	0.17(0.01)	0.44(0.07)	2.07(0.16)
Calvert 1995a	Dobermann	30 - 36%	x	x	x	x	x	x	x
Crippa *et al* 1992	Beagle	40 (9)	77(10)	x	x	x	x	x	x
Morrison *et al* 1992	Pembroke corgi	44*	x	21*	18*	x	x	x	x
Morrison *et al* 1992	Miniature poodle	47*	x	12*	10*	x	x	x	x
Morrison *et al* 1992	Afghan hound	33*	x	26*	26*	x	x	x	x
Morrison *et al* 1992	Golden retriever	39*	x	27*	24*	x	x	x	x
Herrtage 1994	Boxers	33(8)	x	23(2)	22(2)	0.07(0.01)	0.18(0.02)	x	x
Snyder *et al* 1995	Greyhound	29(4.2)	x	x	x	0.0685(0.008)	0.183(0.016)	0.38(0.05)	1.6(0.29)
Pietra *et al* 1998	English Setter	39(10)	x	27(3.9)	20.7(2.4)	x	x	x	x
Vollmar 1996	Irish Wolfhound	35.2 (4.9)	x	33.7 (5.9)	30.5 (4.0)	x	x	x	x
Brownlie, unpublished1 995	Irish Wolfhound - males	28.38(4.71)	x	46.81(5.66)	33.29(3.02)	x	x	x	x
Brownlie, unpublished 1995	Irish Wolfhound - females	27.33(3.78)	x	44.55(5.46)	32.66 (2.07)	x	x	x	x
Bodey (1998).	Scottish Deerhounds	27	x	x	x	x	x	x	x
Borgarelli *et al* 1996	Great Dane	31.4 (4)	57(7)	x	x	x	x	x	x
Tidholm & Jonsson 1996	Newfoundland	>25%	x	x	x	x	x	x	x
Koch *et al* 1996	Newfoundland Irish wolfhound Great Dane	30* (22-37) 28* (20-34) 25* (18-36)	57* (44-66) 54* (38-61) 48* (33-65)	30* (24-33) 31* (22-35) 33* (28-46)	29* (26-33) 30* (29-33) 29.5* (28-34)		0.17* (0.14-0.2) 0.16* (0.14-0.19) 0.15* (0.12-0.18)	x	1.7* (1.1-2.5) 1.7* (1.0-2.2) 1.7* (1.0-2.3)
Mashiro *et al* 1976	Mongrels	x	53.8	x	x	x	x	x	x
Boon *et al* 1983	Various	36.26	x	✓	✓	x	0.179(0.018)	x	2.07(0.37)
Lombard 1984b	Various	39(6)	x	✓	✓	x	x	x	x
Bonagura *et al* 1985	Unspecified	29-45	x	✓	✓	x	x	x	x
Jacobs & Mahjoob 1988b	Mongrels	✓	x	x	x	x	x	x	x
Lusk & Ettinger 1990	Unspecified	39(6)	x	✓	✓	x	x	x	x
DeMadron 1996	Various	30.7(7.6)	60.8(7.0)	✓	✓	x	x	x	2.0(0.4)
Pipers *et al* 1978	Various	x	x	x	x	0.069(0.008)	0.255(0.01)	0.27(0.04)	x
Atkins & Snyder 1992	Various	x	x	x	x	0.054 (0.007)	0.159(0.015)	0.34(0.05)	2.48(0.5)

Table B.1.
M-mode echocardiography. Veterinary literature .....
continued / 2

Author	Breed	Comment on study or limitations with study
Gooding *et al* 1986a	Cocker spaniel	Regression line to show effect of BSA also. Dogs from a DCM kennel - subgroup had low FS%. No repeats.
Calvert *et al* 1982	Dobermann	Minimal data about normal Dobermanns given.
Calvert & Brown 1986	Dobermann	Clinically normal, repeat echo after 1 year.
Calvert 1995a	Dobermann	Criteria for normality in this breed.
Crippa *et al* 1992	Beagle	EF from cubed formula. Regression line to show influence of body weight.
Morrison *et al* 1992	Pembroke corgi	*median shown. Regression lines to show influence of weight. Dogs 2- 7 years old.
Morrison *et al* 1992	Miniature poodle	*median shown. Regression lines to show influence of weight. Dogs 2- 7 years old.
Morrison *et al* 1992	Afghan hound	*median shown. Regression lines to show influence of weight. Dogs 2- 7 years old.
Morrison *et al* 1992	Golden retriever	*median shown. Regression lines to show influence of weight. Dogs 2- 7 years old.
Herrtage 1994	Boxers	Little correlation shown with body weight. Absolute values advocated.
Snyder *et al* 1995	Greyhound	Regression lines to show influence of body weight and BSA, compared with breeds of similar weight (Morrison et al 1992).
Pietra *et al* 1998	English Setter	Apart from LA & Ao, values similar to those predicted from regression lines.
Vollmar 1996	Irish Wolfhound	EPSS 7.4 (1.5). No regression analysis.
Brownlie, unpublished 1995	Irish Wolfhound - males	LA & Ao from 2D. Personal communication.
Brownlie, unpublished 1995	Irish Wolfhound - females	LA & Ao from 2D. Personal communication.
Bodey (1998).	Scottish Deerhounds	No standard deviations. No attempts to separate individuals with heart disease. Actual values not given in reference (personal communication).
Borgarelli *et al* 1996	Great Dane	EF from Teicholz. Published references with regression lines over-estimated LV parameters. FS lower than published values. No follow up. No figures given.
Tidholm & Jonsson 1996	Newfoundland	Included dogs of 3.5 mths and sig no. < 2 yo. 10% normal dogs had FS<22%. Overlap with DCM dogs.
Koch *et al* 1996	Newfoundland Irish wolfhound Great Dane	Results given as Median (5-95 percentiles). Newfs, n=27, IWH, n=20, g.Danes, n=15). Dogs re-evaluated for up to 4 years (but median ages 2.5 - 3.5 years)
Mashiro *et al* 1976	Mongrels	EF using corrected cube method. Correlated SV etc with indicator-dilution technique
Boon *et al* 1983	Various	Regression line to show influence of body surface area. Dogs from 6 mths - 6 yrs old (mean age only 16.4 mths).
Lombard 1984b	Various	Regression lines to show influence of body weight. Dogs 1 - 9 years old.
Bonagura *et al* 1985	Unspecified	Regression lines to show influence of body weight. Data repeated by Miller et al 1989.
Jacobs & Mahjoob 1988b	Mongrels	Regression line to show influence of body surface area and derivative of heart rate (square root of R-R interval)
Lusk & Ettinger 1990	Unspecified	Data obtained from review of literature.
DeMadron 1996	Various	From review of literature and based on weight tables derived from regression equations. EF from Teicholz.
Pipers *et al* 1978	Various	Anaesthetised animals. No correction for heart rate.
Atkins & Snyder 1992	Various	from Atkins, unpublished data.

Although Doppler echocardiography has been available to many veterinary cardiologists for a considerable time, it is surprising that little has been published on Doppler reference values for dogs. Some general articles review the technique and views required to perform an echocardiographic examination (Kirberger 1991b; Darke 1992). Yuill and O'Grady (1990;1991) published Doppler derived peak velocity measurements across each cardiac valve in normal dogs of unspecified breeds, using duplex continuous wave (CW) Doppler and showed that "physiological" pulmonic and tricuspid insufficiency in 70% and 50% of dogs respectively. A similar study was done by Kirberger and colleagues (1992a) who published flow patterns and velocities from normal valves in Beagles and German shepherd dogs using pulsed wave Doppler echocardiography. This latter study can be heavily criticised as suboptimal angles for Doppler were obtained, judging from illustrations provided, and they advocated the use of the angle correction facility to compromise for this technical limitation. However, these latter authors suggested measuring canine aortic flow through the suprasternal notch to attempt to improve the angle, although a stand-alone probe was used and so angle could not be estimated and peak velocity was in fact obtained from an apical view. Results obtained from these two studies are shown in Table B.2.

Table B.2. Reference values of Doppler derived blood flow velocities across normal canine heart valves

	PA(R) (m/s)	PA(L) (m/s)	Ao (L.Ap) (m/s)	MV (E/A) (m/s)	TV (E/A) (m/s)
Yuill & O'Grady 1991	0.98 (0.094)	0.955 (0.103)	1.18 (0.11)	0.86/- (peak) (0.1)	0.69/- (peak) (0.08)
Kirberger *et al* 1992a	1.2 (0.2)	nr	1.57 (0.33)	0.91/0.63 (0.15)/(0.13)	0.86/0.58 (0.15)/(0.16)

Abbreviations: PA(R)= pulmonary artery from right parasternal view; PA(L) = pulmonary artery from left parasternal window; Ao (L.Ap)= aorta from left apical view; MV(E/A) = mitral valve, E and A peaks; TV(E/A)= tricuspid valves E and A peaks; nr = not recorded. Results shown as mean (standard deviation).

Kirberger and colleagues (1992b) reported on the influence of age, heart rate and weight on these variables, showing that increasing heart rate and decreasing mass was associated with increased peak velocities.

B.6. *Current criteria for the echocardiographic diagnosis of canine dilated cardiomyopathy*

The diagnosis of DCM is based on the demonstration of left ventricular enlargement and impaired contractility, and these criteria have remained unchanged since first reported (Bonagura & Herring 1985). However, it is now appreciated that reference values, preferably breed specific, are required, and there are problems in the diagnosis of equivocal disease (Bond 1991). Some criteria used for the diagnosis of DCM in certain breeds are given below in Table B.3.

Table B.3. Criteria used in the diagnosis of canine idiopathic dilated cardiomyopathy by various authors

	Breed	FS%	LV dilation	IVS/LVpw parameters	Other criteria
Calvert 1995a	Dobermann	< 25%	LVIDd > 50 mm	IVSd/LVpwd < 7 mm IVSs/LVpws < 10 mm	x
Monnet *et al* 1995	Various	<25%	LVIDd > 44 mm/m^2	normal or subnormal	presence of CHF
Freeman *et al* 1996	Dalmations/ Dobermanns		"dilated"	x	presence of CHF
Tidholm & Jönsson 1996	Newfoundland	< 22%	x	x	presence of CHF
Tidholm 1996	Various	<25%	x	x	presence of CHF

Abbreviations: as in text. x = not given. CHF = congestive heart failure.

The diagnosis of DCM is not a problem in most symptomatic dogs. There is a major problem in identifying dogs with occult DCM. Equivocal echocardiographic results are common both in normal dogs and in dogs with occult DCM because of inherent limitations in the technique (Calvert 1995a).

Calvert (1995a) found that serial assessment of progressive left ventricular dysfunction was best measured by sequential monitoring of the left ventricular systolic dimension from M-mode rather than fractional shortening percentage.

Different breeds affected by DCM appear to have variable echocardiographic findings specific to their breed. There appears to be a difference in end-systolic volume index or end-diastolic volume index between Dalmations with DCM and Dobermanns with DCM, as well as Dalmations with DCM and other breeds (various) with DCM (Freeman *et al* 1996).

It seems obvious that more sensitive methods of detecting echocardiographic abnormalities are required in dogs with occult DCM. The generation of breed specific reference ranges and the identification of sensitive and specific indicators of impaired systolic or diastolic function will improve the sensitivity of this technique as a screening aid. The Doppler indices, particularly of the aortic ejection phase, accurate methods of assessing ejection fraction and possibly sensitive parameters of diastolic function may prove to be more useful than fractional shortening and chamber size.

B.7.1. *Human echocardiographic criteria for the diagnosis of DCM*

Exclusion of other cardiac disease is important prior to making a diagnosis of idiopathic DCM in man, especially coronary artery disease, myocarditis, hypertension, isolated right ventricular cardiomyopathy etc. (Mestroni *et al* 1994b).

Normally, the demonstration of low ejection fraction is required to make the diagnosis although the actual cut-off value is controversial and ranges from less than 40% (Zimmerman *et al* 1992; Durand *et al* 1995) to less than 55% (Mestroni *et al* 1994b). Left ventricular enlargement is also required with various criteria used to define this, including M-mode parameters of LVIDd exceeding two standard deviations above the mean for weight, age and sex (Zachara *et al* 1993), or LVIDd > 2.7cm/m^2 (based on BSA) (Mestroni *et al* 1994b), or LVIDd exceeding 112% predicted values from multiple linear regression equations (Keeling *et al* 1995; Baig *et al* 1998). Fractional shortening is only rarely used, but values less than 26%

(Zachara *et al* 1993) or less than 25% (Keeling *et al* 1995) are judged to reflect hypokinesis. Lestuzzi and colleagues (1991) corrected LV and LA dimensions for age and BSA. In diagnosis of DCM, Lestuzzi *et al* (1991) arbitrarily set a number of major criteria and minor criteria.

B.7.2. *The problem of recognition and interpretation of mild echocardiographic abnormalities*

Keeling and McKenna (1994) asked whether human individuals with mild echocardiographic abnormalities have early DCM. There is diagnostic difficulty in identification of patients with early DCM; even in man, there are no well defined criteria for diagnosis of early DCM where there is less marked ventricular dilatation or dysfunction. In screening families with DCM, in determining mode of inheritance or utilising molecular genetic techniques, an accurate diagnosis is crucial (Marian & Roberts 1993) and it will be vital in human and canine echocardiography to determine criteria for normality and to interpret the significance of slight abnormalities. In the literature, echocardiography has been suggested as both under-estimating and over-estimating the frequency of DCM during family screening. In the UK families with DCM described by Keeling and colleagues (1995), they reported a rather low frequency of familial DCM (11%) compared with other series (e.g. Goerss *et al* 1995). However, not included in this figure were relatives showing left ventricular enlargement (16%) and relatives with a low fractional shortening $< 25\%$ (4%). The high prevalence of left ventricular enlargement in members of families with familial DCM compared with relatives of non-familial DCM patients certainly supports the possibility that mild left ventricular enlargement may represent early DCM (Keeling *et al* 1995). Baig and colleagues (1998) from this group (St. George's Hospital, London) detailed the cardiac abnormalities in symptom-free relatives of familial DCM patients with serial evaluation over approximately three years. They concluded that over a quarter of these relatives developed more overt evidence of DCM in this time period. Patients with left ventricular enlargement (defined as a left ventricular end-diastolic dimension $\geq 112\%$ over the predicted

value, using the formula dependent on BSA and age of individual patient) had a statistically significant predictive association with the development of DCM. Patients with depressed fractional shortening over the time period of this study did not show the same progression and Baig and colleagues (1998) postulated that these patients may have a limited manifestation of disease. Symptom free relatives may be described as having a latent form of cardiomyopathic involvement or they are at a different stage of DCM (Zachara *et al* 1993), but these authors also postulated that the cut-offs in the definition of normality may be too sensitive and these mild echocardiographic abnormalities do not represent disease; they appreciated the importance of follow up studies on such patients to assess whether they had progressive disease and also in interpreting these "carriers" in pedigrees of affected families in determining the mode of inheritance (Zachara *et al* 1993). The problem of assessing relatives at a single point in time, as is usual in the human family series in the literature (Michels *et al* 1992, Zachara *et al* 1993; Mestroni *et al* 1994b, Goerss *et al* 1995) can lead to underestimation of the frequency of disease (Goerss *et al* 1995; Keeling *et al* 1995). Follow up of relatives with mild left ventricular enlargement will show whether a significant proportion of these will develop progressive left ventricular dilatation and dysfunction, supporting the fact that left ventricular enlargement does represent early DCM (Keeling *et al* 1995) although where this has been done, there does not appear to be an answer to date. Lestuzzi and colleagues (1991) reported on one borderline patient developing the disease but another to normalise. The significance of left ventricular enlargement or a low fractional shortening in a relative of a DCM patient remains to be established (Keeling *et al* 1995).

AN ECHOCARDIOGRAPHIC / DOPPLER EVALUATION OF A POPULATION OF NEWFOUNDLAND DOGS

MATERIALS AND METHODS

B.8. *Aims of this study*

There were three major aims for this part of the study.

(i) To generate breed-specific echocardiographic criteria of Normality in Newfoundland dogs, and to identify which independent variables affect these.

(ii) To investigate various parameters of left sided chamber sizes and systolic and diastolic function, and to compare these in normal Newfoundlands, Newfoundlands with DCM or occult DCM and dogs with other echocardiographic abnormalities. From this information, to define more sensitive or specific criteria for the diagnosis of occult DCM or to identify echocardiographic / Doppler predictors of disease development.

(iii) To investigate the use of serial echocardiographic evaluation of dogs, including a repeatability study and the monitoring of progression of echocardiographic abnormalities.

B.9. *Newfoundlands*

B.9.1. *Criteria for dog selection*

Newfoundlands were chosen because they are known to have increased prevalence of both DCM and subaortic stenosis.

Dogs selected to generate breed specific reference ranges were over eighteen months old (so were judged to be adult size). There was no upper age limit. The date of birth and sex and colour of each dog was recorded. An accurate body weight was determined and recorded for each dog when feasible, otherwise an approximate estimate of the weight based on sex and body condition was noted. A copy of the

pedigree was retained for each dog included for pedigree analysis where indicated. Information about numbers, gender and status of siblings was recorded.

Dogs from lines in which there had been a family history of DCM prompted recording a detailed family history. Details about the cardiac illness of affected members was obtained from the owner or breeder and the veterinary surgeon where this was possible. Where the affected family member was alive and available, it was examined by echocardiography / Doppler to confirm the diagnosis if owner consent was given. As many relatives from such a family were screened as possible, across as many generations as possible.

B.9.2. *Examination of each individual dog*

A brief general history was recorded for each dog.

A detailed clinical examination referable to the cardiorespiratory system was made. Each dog was carefully auscultated at rest in a quiet environment, using a Littman Cardiology II stethoscope (3M). Note was made of heart rate and the degree of excitement or stress the dog showed. Any heart murmur was localised and graded (out of VI), with note made of its character, radiation, duration and location in the cardiac cycle. The presence of abnormal heart sounds (e.g. diastolic gallops) was recorded. The presence of any arrhythmia was noted, and documented with a six lead electrocardiogram according to standard practice (Tilley 1992a;b) using a single channel portable electrocardiography machine (Cardiofax^{R}; Nihon Kohden).

B.10. *Echocardiographic / Doppler examination*

B.10.1. *Patient preparation*

The hair coat was clipped over the site of precordial impulse on the left and right hemithoraces and an area of ventral midline immediately caudal to the xiphisternum.

Dogs were placed on a purpose designed table in lateral recumbency on a comfortable bedding material. The owner was involved in restraining the dog, so most dogs were very relaxed and in some cases fell asleep during the procedure. The area was prepared by spraying with surgical spirit and applying ultrasound gel (Henley's Medical Ultrasound Gel; Brownfields, Welwyn Garden City, Hertfordshire, UK). A simultaneously recorded electrocardiogram was obtained after application of adhesive electrodes (Blue Sensor, Medicotest, Rugmarken, Denmark) to the metacarpo-phalangeal pads on three feet and secured with tape. Lead II was normally recorded if ECG quality on the screen was adequate (right fore and left hind) with the third electrode acting as the earth lead.

B.10.2. *Echocardiograph machine and set up*

The echocardiography machine was the Esaote SIM 7000 Challenge (Esoate Biomedica, Firenze, Italy) which had annular array probes with dynamic focusing. Normally a 2.5 - 3.5 MHz transducer was used for Newfoundlands, imaging at 2.25 or 3.5 MHz with Doppler at 2.0 or 2.25 MHz. Appropriate sections of the scans were recorded by videocasette recorder (SVO-9500MDP; Sony Corporation, Japan) on Super VHS video tapes (Panasonic SVHS; Matsushita Electric Industrial Co. Ltd., Japan).

The depth of field was set to include the entire heart on the screen and so was individualised for each dog. The imaging angle was normally 75° although where recordings for left ventricular and left atrial areas were required, this was increased to 90°. Overall gain and time gain compensation settings were set for each dog to give good image quality, where endocardial surfaces could be delineated, but gain was minimised to allow for optimal colour flow Doppler echocardiography (CFDE).

Once set, every attempt was made not to alter the settings. During CFDE, the angle of colour flow was 20° and the velocity variance map 1 was used, which gave an indication of velocity of blood towards or away from the transducer with varying intensity or red or blue respectively, and areas of turbulent flow were encoded green. The variable focus indicator was positioned at the level of the area to be interrogated. Colour gain was set to just below that where artefactual speckling appeared due to over-gain. Once set, every attempt was made not to alter it during a scan. When M-mode recordings were made, the M-mode cursor was positioned guided by the two-dimensional images. During pulsed wave spectral Doppler recordings, sample volume size was normally four millimetres and low pulse repetition frequency was used and every attempt made to align blood flow in the area to be interrogated to be parallel to the Doppler cursor. A spectral signal was displayed at maximal sweep speed (1 sweep every 2 seconds = 100 mm/s) and the baseline position and velocity scale was altered to display maximal envelopes. The Doppler gain and filtration was set to be the minimal required for good well defined envelopes. Weak signals occasionally required to be improved by reducing the Doppler frequency or increasing sample volume size (to six millimetres). Occasionally, the power and reject facilities were altered. If the Nyquist limit was exceeded, either high pulse rate repetition frequency was used or the blood flow velocity spectrum was recorded by continuous wave Doppler.

B.10.3. *Images obtained and recorded*

The following images were obtained and recorded.

From a caudal right parasternal four chamber view, the left ventricular length and area was maximised and recorded for a number of cardiac cycles. Slightly more cranially, the four chamber view was repeated with maximised left atrial area which was recorded. CFDE was used with a velocity variance map to investigate and record blood flow through the mitral valve and the aortic valve. The interatrial and interventricular septae were also scrutinised. A four chamber long axis view was

obtained, ensuring that the left ventricular axis was as perpendicular to the ultrasound beam as possible, giving a horizontal image.

This was changed to a caudal parasternal short axis view of the left ventricle by 90° transducer rotation, and the M-mode cursor was positioned through the centre of the chamber at the level of the chordae tendinae, ensuring that the left ventricular cavity was symmetrical and rounded for optimal M-mode positioning. A left ventricular M-mode was recorded. At the level of the mitral valve, a mitral valve M-mode was recorded, ensuring that both anterior and posterior mitral valve leaflets were included.

A cranial long axis view allowed 2D and CFDE assessment of the left ventricular outflow tract, aortic valves and proximal aorta. A cranial short axis view was obtained at the level of the aortic valves including the left atrium in short axis, with the left auricular appendage. The left atrium was maximised in this view without losing the aortic symmetric cross sectional image. This two dimensional view was recorded, to enable two dimensional measurements of the aorta and left atrium to be made, according to the technique described by Häggström *et al* (1994). The M-mode cursor was then positioned to allow an M-mode recording of the aortic root with aortic valves and the left auricular appendage. The cranial short axis view of the aorta was repositioned to maximise the pulmonary trunk, and angled to allow the trunk to be as parallel as possible to the colour flow and spectral Doppler angle. CFDE of blood flow in the right ventricular outflow tract and pulmonary trunk was assessed, following which the sample volume (set at four millimetres) was positioned just beyond the pulmonic valves. Pulsed wave Doppler recording at fastest sweep speed was made and attempt made to obtain clean spectral envelopes with minimal spectral dispersion.

With the dog still in right lateral recumbency, a subcostal view of the heart was obtained, with the left ventricular outflow tract and the aorta visible and positioned to be as parallel as possible to the Doppler cursor. In large dogs, pulsed wave Doppler

resulted in the Nyquist limit usually being exceeded due to the depth of the sample volume, requiring the use of continuous wave Doppler spectra to display aortic outflow. Fastest sweep speed was used and normally, machine settings had to be altered to obtain a signal of adequate diagnostic quality, which was recorded.

In left lateral recumbency, an apical four chamber view was obtained. The image was optimised by attempting to make it as vertical as possible, with maximal left ventricular length and area. The transducer was slid as near the sternum and the apex of the heart as possible to achieve this without sacrificing image quality. Transducer rotation of 90^{o} allowed a two chamber view to be displayed. The aorta could be imaged from either a two or four chamber view, giving the so-called three or five chamber views. Optimal alignment with aortic outflow was determined from the two dimensional images. From the four chamber view, CFDE of the mitral valve was recorded. The sample volume was positioned just between the tips of the leaflets of the mitral valve and mitral inflow recorded. If mitral regurgitation had been identified on CFDE, continuous wave Doppler was used to attempt to record the mitral regurgitation spectrum. From an apical five chamber view, colour flow mapping indicated the region where both mitral inflow (red) and left ventricular outflow (blue) was present. The sample volume was positioned in this area to obtain a spectral recording of simultaneous mitral inflow and left ventricular outflow, with as clear a definition as possible of the end of the outflow trace and the start of the inflow trace, which indicated the isovolumic relaxation time. The left ventricular outflow tract and aorta were then aligned from a three chamber or a five chamber apical view to allow CFDE and spectral Doppler recording of aortic outflow. The sample volume was positioned just beyond the aortic valve leaflets. From the four chamber view with the atrioventricular annulus as perpendicular as possible to the M-mode cursor, mitral annulus motion of the septal and the lateral annulus was recorded. From the four chamber view, the entry of pulmonary veins into the left atrium were maximised, by altering the focus and using colour flow guidance if effective at this depth. A pulmonary vein was selected which was parallel to the Doppler cursor (usually the right middle lobe pulmonary vein). The sample volume

(as small as possible, usually 4 mm) was positioned about 0.5 cm within a pulmonary vein and a low pulsed repetition frequency recording of pulmonary venous inflow was made at high sweep speed. Normally, the gain had to be increased and the filtration minimised to obtain an adequate signal. Settings were altered to allow a recording which was of good quality as possible, preferably showing laminar flow (although this was not always possible). An attempt was made during the recording to recognise the three distinct waves of pulmonary venous inflow. Repositioning within the same pulmonary vein or another vein was made until an adequate signal was obtained.

From a left parasternal four chamber view, CFDE and spectral Doppler recording of tricuspid inflow was obtained. Any tricuspid regurgitation was recorded. From the cranial left parasternal view with the short axis aorta, the pulmonary artery was displayed by CFDE and spectral Doppler recording.

B.10.4. *Analysis of the echocardiography / Doppler recording*

B.10.4.1. *General methods*

Full echocardiographic analysis was done off-line from the video cassette recordings. Diastolic events were timed from the ECG as the start of the QRS complex. Systolic events were timed from the ECG as the end of the T wave or were determined from the mechanical phase of the scan itself. For each parameter, at least five measurements were obtained for patients in sinus rhythm without accentuated sinus arrhythmia. Events associated with ventricular or supraventricular ectopy were ignored. Where there was marked sinus arrhythmia or atrial fibrillation, up to ten measurements were obtained. As far as possible, five to ten consecutive beats were assessed. During the analyses, the approximately average heart rate during each batch of measurements simultaneously calculated from the ECG and recorded on the images. Occasionally, the heart rate mean was calculated from a group of R-R interval measurements from the ECG, dividing the mean result by 60. This was necessary in certain instances, such as dogs with tall T waves with low voltage QRS complexes. All data were stored on an Excel (v7; Microsoft Inc.) spreadsheet). Two-

dimensional echocardiographic parameters were measured using trailing-edge to leading-edge of endocardial borders (O'Grady *et al* 1986), using the black-white interface to guide measurements (Schiller *et al* 1989; Feigenbaum 1994c). M-mode parameters were normally measured using the leading-edge to leading-edge method advocated by the American Society for Echocardiography (Sahn *et al* 1978), although for the aortic diameter (diastole) and left atrium (systole), internal diameters were selected instead. Since Newfoundlands tended to image poorly and usually had an appreciable amount of periaortic fat, it was considered that internal dimensions were more likely to be reproducible, and they allowed comparison with the two-dimensional measurements of these parameters. For Doppler parameters, peak velocities were measured, although velocity time integrals were traced around the modal velocities, according to Feigenbaum (1994c). If there was not a clear definition between two waves that were to be measured (e.g. close E and A waves on mitral inflow), a perpendicular line was dropped to the baseline on the spectral Doppler tracing to allow time durations or flow integrals to be measured.

B.10.4.2. *Right parasternal view measurements*

B.10.4.2.1. *Left ventricular volumes and ejection fraction from a right parasternal view*

From the right parasternal four chamber long axis view maximising the length and area of the left ventricle, a diastolic frame was frozen and the left ventricular area was traced, within the endocardial border using the tracker ball. Papillary muscles were ignored. At the base of the left ventricle, the area was closed at the level of the mitral annulus. From the centre of the annulus at the level of this closure line, the length of the left ventricle was determined to the apex. The software calculated the left ventricular diastolic volume by the length-area method. For each diastolic frame, the subsequent systolic frame was selected (end of T wave) and the left ventricular area and length in systole was determined and the volume calculated by the machine. Record was made of LV areas and lengths, as well as the left ventricular volume derivatives. From each pair of diastolic and systolic measurements, as well as the end diastolic volume (LVdv) and the end systolic volume (LVsv) calculations, the stroke

volume (SV) and the ejection fraction (EF) were automatically calculated and displayed by the software following the volumetric computations.

B.10.4.2.2. *Determination of left atrial parameters from a right parasternal view*

The slightly more cranial long axis four chamber view with a decreased image angle to increase the frame rate was used to assess the left atrium. A technique to assess change in left atrial area was sought to attempt to correlate this with pulmonary venous inflow. Measurements were obtained during ventricular diastole and systole guided by the ECG. In systole, the area of the entire left atrium up to the closed mitral valves, and tracing straight across the pulmonary veins was used. In diastole, as the left atrium functions as a conduit to the left ventricle and the mitral valves are open, it was decided to close the area in a line drawn straight across the mitral annulus. Left atrial width in ventricular systole and diastole was also obtained parallel to but just above the mitral annulus, in order to attempt to document "stretch" of the mitral annulus and any secondary mitral regurgitation associated with dilated cardiomyopathy.

Calculation of percentage change in LA area from the 4 chamber long axis view was done (similar to the diastolic emptying index described by Clarkson and others (1995) to give a left atrial emptying index (LAEI).

B.10.4.2.3. *CFDE screening*

CFDE was used to assess the presence of any mitral, tricupsid or aortic incompetence from a four chamber or a five chamber RPS or left apical views, and pulmonic valves were assessed from left and right parasternal cranial short axis views. Any valvular incompetence was subjectively graded as a trace, 1+, 2+, 3+ or 4+ depending on jet area, relative to receiving area and jet width at its origin, simplifying the methods described by a number of authors, attempting to mimic the Seller's angiographic classification (Helmcke *et al* 1987; Perry *et al* 1987; Perry 1989; Keren & LeJemtel 1989).

B.10.4.2.4. *Left ventricular M-mode*

The left ventricular M-mode study was measured using the leading edge - leading edge technique (Sahn *et al* 1978). Diastole was determined at the start of the QRS complex. Systole was defined as the nadir of the septal posterior motion. The right ventricle in diastole was measured, although in many cases was unreliable as the artefacts in the near field made endocardial surface of the right ventricle difficult to determine. The interventricular septum was calculated in diastole (IVSd) and systole (IVSs). The left ventricular internal dimension was measured in diastole (LVIDd) and systole (LVIDs). The left ventricular posterior wall was measured in diastole (LVpwd) and systole (LVpws). Because systole was defined as the nadir of septal motion, and the free wall sometimes lagged behind the septum, the systolic dimension of the left ventricle and the systolic thickness of the left ventricular free wall was underestimated. The software computed the fractional shortening (FS%), and the percentage thickening of the septum (%thIVS) and the left ventricular posterior wall (%thLVpw). The ejection fraction was also calculated by the machine software, using Teicholz method, and was recorded.

B.10.4.2.5. *Mitral M-mode*

From the mitral M-mode, the E point to septal separation was measured.

B.10.4.2.6. *Left atrium to aortic root ratio (two-dimensional measurement)*

From a cranial short axis view of the left atrium showing the left auricular appendage and the aortic valves, diastolic dimensions of the aortic root and the maximal left atrium in the same plane was measured (Häggström *et al* 1994). The maximal plane of the left atrium often corresponded to entry of pulmonary veins - these were ignored and the border extrapolated to measure the posterior limit to the left atrial measurement. A two dimensional ratio of left atrium to aorta was calculated.

B.10.4.2.7. *Aortic M-mode*

From the aortic M-mode, a subjective impression of the anterior systolic excursions of the aortic root was assessed. The aortic root was measured in diastole (start of QRS complex) and the left auricular appendage in systole (maximum), measuring internal diameters rather than leading-edge to leading-edge techniques. The M-mode ratio of left atrium to aorta was calculated.

B.10.4.2.8. *Pulmonary artery*

A spectral Doppler trace of pulmonary outflow was measured if the envelopes were of good diagnostic quality. The peak pulmonary artery velocity obtained.

B.10.4.3. *Subcostal view measurements: aortic flow*

The aortic outflow velocity was almost always maximal from the subcostal position. Because of the depth of field, normally a continuous wave spectral signal was obtained. Parameters measured were:

(i) Peak velocity (Aov).

(ii) The velocity time integral (Aovti) was measured by tracing around the spectrum, to allow calculation of area under the curve by the machine.

(iii) Acceleration was assessed according to the steepest slope of the acceleration phase of the signal (dv/dt_{max}) and the slope from start on outflow to peak flow, to give mean acceleration (dv/dt_{mean}). Acceleration time was measured as the interval between the two points used to calculate dv/dt_{mean}.

(iv) Systolic time intervals were measured from the Doppler aortic signal. Pre-ejection period (PEP) was measured from the start of the QRS complex to the onset of aortic flow at the baseline, ignoring any low velocity, poorly defined initial signal. The left ventricular ejection time (ET) was measured as the duration of the aortic spectrum at the baseline, again ignoring any low velocity signals. From these measurements, mean values were calculated and used to calculate a pre-ejection period: ejection time ratio (PEP:ET). The velocity of circumferential fibre shortening (Vcf) was calculated by dividing the fractional notation of the mean fractional shortening obtained by M-mode by the mean ejection time.

(v) Parameters which were likely to be influenced by heart rate were normalised for the R-R interval by dividing by the square root of the R-R interval. These included PEP, ET, acceleration time, PEP:ET ratio and Vcf.

A normal peak aortic velocity was defined as <1.7 m/s (Lehmkuhl & Bonagura 1995). Dogs with aortic velocity in excess of this were defined as having mild sub-aortic stenosis, particularly if there was other evidence of left ventricular outflow tract abnormalities such as two-dimensional lesions, turbulent blood flow or step up of velocity in the outflow tract, or aortic regurgitation (Lehmkuhl & Bonagura 1995).

B.10.4.4. *Left parasternal view measurements*

B.10.4.4.1. *Mitral inflow*

The presence of any mitral regurgitation was again assessed by CFDE as previously described.

Mitral inflow was assessed and the following measurements made:

(i) Maximal velocity of E (Ev) and A (Av) waves, by measuring peak velocity. The mitral E:A velocity ratio was calculated.

(ii) Velocity time integral (vti) of E (Evti) and A (Avti) waves. The envelopes were traced along the modal (brightest) velocity spectrum (Mantero *et al* 1995). With slow heart rates, if there were late diastasis phases to mitral inflow, these were ignored. The E:A velocity time integral ratio was determined. Evti and Avti were added to determine the total forward mitral inflow (ignoring any contribution in diastasis).

(iii) The proportions of early (and late) left ventricular filling of total mitral inflow was calculated by dividing Evti (or Avti) by the total mitral vti.

(iv) Mitral E and A wave velocities were normalised by dividing by the total mitral vti (Bowman *et al* 1988; Santilli & Bussadori 1998).

(v) The duration of E (Edur) was measured as the duration of the E envelope. The duration of the A wave (Adur) was measured in a similar way. With fast heart

rates, if E and A peaks were summated, a line perpendicular to the baseline was dropped between the two peaks at their intersection.

(vi) The deceleration time of E was assessed (E decel), by measuring the time interval from the peak of the E to the intersection of the E with the baseline.

(vii) Time intervals which are influenced by heart rate were normalised by indexing to the square root of the R-R interval. These included E duration, E deceleration time and A duration.

B.10.4.4.2. *Mitral regurgitation*

If mitral regurgitation was identified and a good quality spectral continuous wave (CW) Doppler signal could be achieved, this was recorded to allow calculation of dP/dt, based on the techniques described (Bargiggia *et al* 1989; Chen *et al* 1991). From the spectrum, a velocity measuring cursor was used and positioned at approximately one metre per second on the acceleration edge of the spectrum. The corresponding pressure was displayed by the machine soft ware, using the modified Bernouille equation. A second velocity cursor was positioned near the peak of the acceleration slope (usually between three and four metres per second). The corresponding pressure was recorded and difference in pressures between the two measurements was calculated. A time cursor was used to measure the interval between the two velocity cursors. The difference in pressures was divided by the time to give a rate of change of pressure (dP/dt). The negative rate of pressure change (-dP/dt) could be calculated in a similar manner, but during this study, where no dog had severe mitral regurgitation, it was unusual to obtain clear cut diagnostic deceleration edges of the mitral regurgitant signal, and this was not done.

B.10.4.4.3. *Isovolumic relaxation time*

If an adequate spectral signal was obtained from the left ventricle showing both mitral inflow and left ventricular outflow, the time between closure of aortic valves and opening of mitral valves was measured, giving the isovolumic relaxation time (IVRT). This was normalised to heart rate by dividing by the square root of the R-R interval (IVRT/$\sqrt{}$R-R).

B.10.4.4.4. *Aortic outflow*

The cranial left parasternal view with the aorta in long axis was scrutinised to identify any two dimensional abnormalities of the left ventricular outflow tract or aortic valves, and CFDE recordings were assessed for the presence of aortic regurgitation or any variance indicating turbulent flow in the left ventricular outflow tract or the aorta.

From the apical three or five chamber view, the angle of aortic flow was assessed compared with the cursor. If this was parallel or within 20^{o}, and a good quality spectral envelope was recorded, the maximal velocity was measured. Normally, it was less than that obtained from the subcostal view (showing that the subcostal view is usually more successful at aligning the Doppler cursor parallel with aortic flow) (Lehmkuhl & Bonagura 1994). If the apical aortic flow velocity was higher than that obtained from the subcostal view or if a subcostal aortic Doppler spectra were non-diagnostic, the previously mentioned aortic parameters were also measured.

B.10.4.4.5. *Mitral annulus motion*

Septal and lateral (posterior) mitral annulus motion (MAMseptal and MAMlateral respectively) was measured from M-modes using the maximal excursion between end diastole and systole. This gives an indication of apico-basilar contractility. The mean of these two values gave the mean mitral annulus motion (MAMmean) from the two measurements derived from the left apical four chamber view.

B.10.4.4.6. *Pulmonary Venous Flow*

From the recording of PVF, the following parameters were measured:

(i) Velocity of systolic (S), diastolic (D) and atrial reversal (Ar) waves and R_2 waves. The S:D velocity ratio was calculated.

(ii) The velocity time integral of S (Svti) and D (Dvti) was measured by planimetry after tracing with a tracker ball around the corresponding envelopes. If there was overlap between S and D, a perpendicular line was dropped to baseline between

these and used the differentiate between the two forward waves. The presence of R_2 helped to distinguish between the two waves. The S:D velocity time integral ratio was determined. Calculation of the systolic fraction of total forward flow (Svti/(Svti+Dvti)) was made.

(iii) Durations of S, D and Ar and R_2 waves were measured at the baseline where each envelope intersected. Where S and D waves overlapped, a vertical line was dropped at their intersection to the baseline and this was the point used for measurement of durations. At this point, a small reversal wave (R_2) was present normally which could be used to confirm timing. The D deceleration time was measured from the peak velocity of the D wave until the point at which the flow declined to baseline.

(iv) Durations of the pulmonary venous flow parameters are influenced by heart rate and so were indexed to $\sqrt{}$R-R interval.

B.10.4.4.7. *Tricuspid inflow*

The velocity of the E and A waves was measured (TEv and TAv). The tricuspid E:A velocity ratio was calculated. CFDE was used to assess any tricuspid regurgitation.

B.10.4.4.8. *Pulmonary artery (left cranial parasternal view)*

If the pulmonary artery signal was of good quality, the maximal velocity of the pulmonary flow spectra were measured.

B.10.5. *Statistical analyses*

Means and standard deviations were calculated for each parameter using the Excel (v.7) spreadsheet functions.

B.11. *Identification and correction of a software problem*

During the course of this study, a software problem was identified resulting in a time delay in displaying spectral Doppler signals after the ECG, which resulted in prolonged PEP and abnormal PEP:ET ratio. The investigations into this problem are

detailed in the Supplement (available from the author). Echocardiographic data for PEP was corrected by subtracting 38 milliseconds from the time measured.

B.12. *The Newfoundland Study: Body weight and surface area*

B.12.1. *Weight*

Where the body weight was assessed or reported by the owner or estimated with some degree of confidence, these were recorded in the spreadsheet. From the weights of the male and female dogs, means and standard deviations were recorded.

B.12.2. *Body Surface Area*

The body surface area (BSA) was derived from the body weights by the formula:

$$BSA = 10.1 \times [Wt\ (grams)]^{0.667} \times 10^{-4}$$

where BSA = body surface area in m^2, Wt = body weight in grams. (Appendix in Kirk's Current Veterinary Therapy XII. Small Animal Practice. Ed. J.D. Bongura. W.B. Saunders. Philadelphia p. 1416).

B.13. *Categorising Newfoundland dogs into groups*

Summary data (means and derived calculations) for each scan were transferred into a new Excel spreadsheet into sub-divisions based largely on the M-mode data. Dogs with DCM (overt or occult) showed altered left ventricular geometry (increased sphericity of the chamber), may have had arrhythmias, may have had left or biatrial enlargement and usually had left ventricular enlargement with hypokinesis (FS usually < 22%) (DCM group). Dogs with depressed fractional shortening (<18%) were selected by this criterion alone, if they had no additional evidence of DCM prompting their inclusion into this former category (dFS<18% group). Dogs in an equivocal category, with fractional shortening between 18 and 20% were included in a separate division (dFS18-20% group). Left ventricular enlargement was defined as LV M-mode diastolic dimension of >55 mm (males) or >50 mm (females) (LVE

group). Dogs with a subcostal or peak aortic velocity of >1.7 m/s were included in another division, with the overt aortic stenosis cases (SAS group). Each scan was not included into more than one category, although dogs with serial scans may have appeared in different categories depending on the results of the analysis of each scan.

Once echocardiographic reference data had been obtained from the Newfoundlands defined as Normal, limits exceeding and less than mean plus or minus two standard deviations were recorded, and values out with these limits were indicated in bold for each of the Newfoundland categories.

B.14. *Regression analyses and derived calculations in Normal Newfoundlands*

Only nine Newfoundlands over eight years old could be defined as echocardiographically normal. Consequently, data were compared between this normal group and normal Newfoundlands less than eight years old. The two groups were then combined. Descriptive statistics from the entire group were determined, including means, standard deviations, maximum and minimum data points, the 95% confidence interval of the mean and the lower and upper confidence limits. These analyses were performed using SigmaStat (v.2.03; SPSS Inc.).

Variables which were considered to potentially affect the prediction of an echocardiographic parameter were body weight, BSA, age, gender and mean R-R interval recorded at the time of acquisition of the particular parameter. Since mean heart rates, rather than R-R intervals, had been recorded during the echocardiographic quantification, the mean R-R interval was calculated by dividing 60 by the heart rate in beats per minute. Gender was indicated numerically, with 1 = male and 2 = female.

Derived parameters included in the analysis were end-diastolic and end-systolic volume indices (EDVI and ESVI respectively), obtained from indexing the volumes calculated by the single plane ellipse method to BSA. The stroke volume index (SVI) was calculated in a similar manner. All M-mode parameters were also indexed to

BSA. The M-mode left ventricular posterior wall was divided by the chamber diameter diastolic measurements (LVpwd/LVIDd). To attempt to define an "index of sphericity", the left ventricular diastolic length from the right parasternal long axis view (LVld) was divided by the M-mode left ventricular internal diameter in diastole (LVIDd). The ratio of the left atrium to aortic diameter was calculated for both the 2D short axis view measurements and the M-mode measurements. A left atrial emptying index (LAEI) was calculated by dividing the difference between left atrial area in systole and diastole by the left atrial systolic area ((RPSLAas - RPSLAad)/RPSLAas).

Derived parameters from mitral inflow measurements were also included in the analysis. The mitral E:A velocity and velocity time integral ratios were calculated. The peak filling rate indexed to mitral stroke volume was calculated as described by Bowman and others (1988). This was calculated as the peak E velocity divided by the total mitral velocity time inflow integral. In this case, since the total forward vti had not been measured by planimetry, but E and A waves had been measured separately, the sum of Evti and Avti were used, although it was appreciated that any diastasis flow was ignored. As well as the standard parameters of pulmonary venous flow, the ratio of the S and D wave velocities and velocity time integrals was determined. The total forward flow was determined by adding the Svti and Dvti, and the fractional systolic flow was calculated by dividing Svti by this total.

Initial screening linear regressions were carried out to ensure there was a linear relationship between the echocardiographic parameter and each independent variable, and a scatter plot of the residuals (Y axis) was made to ensure that they were evenly distributed around the X axis values.

Forward stepwise regression was then carried out for each echocardiographic parameter (dependent variable) using the independent variables to identify which of these significantly contributed to the dependent variable. If just one independent variable significantly contributing to the dependent variable, linear regression was

run. If more than one independent variable was significantly important, then multiple linear regression was run. The least squares method of linear regression was used. Statistical analyses were all carried out in SigmaStat (v.2.03; SPSS Inc.). The regression equations, the R value (correlation coefficient), which indicates the strength of the relationship between the variables, the R^2 value, the coefficient of determination, a measure of goodness-of fit of data to the regression line, the adjusted R^2 value (corrected for degrees of freedom) and the significance level (p value) were all recorded.

Where body weight and BSA significantly affected the echocardiographic parameter, regression equations were determined for both, for use in generation of reference values for this breed.

Graphs of the dependent variable (echocardiographic parameter) against one of its independent variables show the regression line, 95% confidence intervals for this line and 95% prediction intervals (i.e. 95% of future subjects should be within these lines). These were drawn using the facility in SigmaStat (v.2.03; SPSS Inc.).

B.15. *Comparison between the Newfoundland categories*

The Newfoundland categories were:

(i) the Normal group (dogs of all ages)

(ii) the dilated cardiomyopathy (DCM) group

(iii) two depressed fractional shortening (dFS) groups

(a) one with FS less than 18% (dFS<18%)

(b) the other with FS between 18 and 20% (dFS18-20%)

(iv) the left ventricular enlargement (LVE) group

(v) and the group with (sub)aortic stenosis, with peak aortic velocities exceeding 1.7 m/s (SAS).

B.15.1. *Data analysis*

Statistical analysis was performed using SigmaStat (v.2.03 SPSS Inc.) software. A p value of <0.05 was accepted as being statistically significant. Descriptive statistics for each Newfoundland category were applied. The χ^2 test was used to compare the gender ratios in each category with the total Newfoundland population. ANOVA or ANOVA on ranks (if data not normally distributed or variance was unequal) was used to identify any differences in age, weight, BSA or heart rate between the groups. If differences were identified, the Tukey test or Dunn's method (non-normal data or unequal variance) for multiple pair-wise comparisons were used to identify the significantly different groups.

ANOVA or ANOVA on ranks with the Tukey test or Dunn's method of comparisons were used to assess for statistically significant differences between the groups for each echocardiographic parameter. Some parameters were indexed to BSA. Where differences in heart rate between the groups may have explained some of the differences, the parameter was normalised by dividing by the square-root of the R-R interval.

The "index of sphericity" (2D LVld divided by M-mode LVIDd) was calculated for comparison between groups, in a method similar to that described by Douglas and colleagues (1987b) or D'Cruz and others (1992). The M-mode left ventricular posterior wall diastolic thickness (LVpwd) was indexed to the M-mode LVIDd to allow group comparisons. The mitral EPSS was indexed to the LVIDd for further analysis of differences between groups. The M-mode ratio of LAs: Aod was recorded for analysis.

To display differences between groups, box and whisker plots were used. The central line indicated the median value, the upper and lower limits of the box incorporated data between the 25^{th} and 75^{th} percentiles, and the Whiskers defined the 10^{th} and 90^{th} percentiles. Data points out-with these boundaries were also illustrated.

The number of dogs with mitral regurgitation in each group were noted with the severity, defined by colour flow echocardiography (ranging from 1+ to 4+) graded as absent (0), a trace (0.5), very mild (1), mild (2), moderate (3) or severe (4). χ^2 analysis was used to compare groups. Whether the grade of mitral regurgitation correlated with any of the two-dimensional left atrial parameters was ascertained using stepwise regression and linear regression.

In a similar way, dogs with aortic or pulmonic insufficiency or tricuspid regurgitation were graded and χ^2 analysis was used to compare groups.

If good quality CW spectral Doppler signals of mitral regurgitant jets were obtained, with clean acceleration, the left ventricular dP/dt was calculated according to the methods previously described, and results were compared between individuals.

Newfoundlands with aortic velocities exceeding 1.7 m/s (SAS group) were further analysed. The aortic velocity from the subcostal view was regressed to the M-mode and the 2D left ventricular parameters in an attempt to identify any significant relationship.

Linear regression was also used to investigate the relationship between peak pulmonic velocity (from the left parasternal view) and aortic velocity (from the subcostal view) in the SAS group. Data from all Newfoundland groups was combined to investigate this relationship in the general Newfoundland population.

B.15.2. *Sensitivity and specificity of systolic time intervals in distinguishing between Normal and DCM Newfoundlands*

The systolic time intervals PEP and PEP:ET ratio were used to investigate the sensitivity and specificity of these parameters in differentiating between Normal Newfoundlands and Newfoundlands with DCM. The corrected PEP data was used. The cut-off values selected were based on mean plus two standard deviations (sd)

(mean +2sd) or mean +1 sd for each parameter, where these values had been obtained from the combined Normal Newfoundland group. The normal mean $\pm$ sd PEP was 0.072 $\pm$ 0.010 seconds, with upper limits of normality defined as 0.092 seconds (mean + 2sd) or 0.082 seconds (mean +1sd). The normal mean $\pm$ sd PEP:ET ratio was 0.403 $\pm$ 0.057, with upper limits of normality defined as 0.517 (mean +2sd) or 0.460 (mean +1sd). Dogs in the combined normal groups or the DCM group were separated as having a positive or negative PEP or PEP:ET ratio based on these limits. It was assumed that the diagnosis of normality and DCM was correct for the purpose of this part of the study. The sensitivity was defined as the proportion of DCM cases which were positively identified by these tests and the specificity was defined as the proportion of Normal dogs which were correctly identified by the test.

The positive predictive value of the test was defined as the proportion of Newfoundlands with a positive result which were correctly identified as having DCM. The negative predictive value of the test was the proportion of dogs with a negative result which were correctly identified as being Normal.

This investigation was undertaken to attempt to identify which of PEP or the PEP:ET ratio was most sensitive at distinguishing between Normal and DCM dogs, and which cut-off value resulted in optimal sensitivity and specificity. The selected criterion for an abnormally prolonged PEP:ET ratio of >0.460 was used to identify the numbers of dogs in all other Newfoundland categories with this result.

A similar approach could not be used for other conventionally used parameters to differentiate between Normal and DCM patients, such as left ventricular parameters or fractional shortening, since these parameters had been used to define the Newfoundland categories.

B.16. *The influence of independent variables on the echocardiographic parameters for each Newfoundland group*

Forward stepwise regression was used to identify the influence of the independent variables weight, BSA, age, gender or R-R interval on the echocardiographic variables using similar methods to those described for the Normal Newfoundland group, applying this to all other Newfoundland groups.

B.17. Repeatability study

Since echocardiography / Doppler is used to serially evaluate Newfoundland dogs in this study, it was necessary to investigate the repeatability of these measurements. The study was carried out in boxer dogs. Details of these investigations are not shown but are available in the Supplement (available from the author).

B.18. *Serial scans in the Newfoundland population*

Fifty four dogs had one repeat scan, of which four dogs had a second repeat scan. The scans were performed and the scan categorised for the individual dog as previously described. The category of each scan was indicated; some dogs changed categories by the time of the second scan. Normally, the "final" category was that of the last scan, unless it was felt that there was information in previous scans of better diagnostic quality.

The serial scans for each individual were collated in an Excel (v7; Microsoft Inc.) spreadsheet, included by the final category. The DNA identity and P number (for the Pedigree/Draw data; see later) and the number for each scan were recorded. The time interval between each scan was recorded in months. Any significant event, such as death, was also recorded as time after initial scan. The gender, weight, BSA, presence of any heart murmur or abnormal rhythm and status or comments about each individual were recorded.

B.18.1. *Data analysis*

For each echocardiographic parameter from each scan, the mean and standard deviation (sd) were recorded, having been calculated in the previous spreadsheet (raw data not shown).

As an indicator of the variance for each scan parameter, the standard deviation was presented as a percentage of the mean (sd%Mean), to give a coefficient of variation for each individual scan. The difference between the means (%diffMeans) was calculated as:

$$\%\text{diffMeans} = \frac{\text{Mean 1 - Mean 2}}{\text{Mean 1}} \times 100\%$$

Mean 1 was the mean value for a particular echocardiographic parameter at the initial scan, and Mean 2 was the mean value for that parameter at the second scan, or final scan if the dog received more than one repeat scan.

If the percentage difference between the means (%diffMeans) was greater than any of the percentage standard deviation of the means (sd%Mean) for a particular parameter during either or any of the scans, this was indicated in bold type.

A second method of identifying significant differences between the data sets used to generate each mean was the Student's *t* test (Excel (v7; Microsoft corporation)). Using the raw data for each echocardiographic parameter, data from the initial scan was compared with the data from the second (or final) scan under the null hypothesis that there are no significant differences between the data sets in a dog with no cardiac disease or no significant progression of cardiac disease between the first and second (or final) scans. The levels of significance were $p<0.001$, $p<0.01$, $p<0.05$ or *ns* (not significant), and the *t* test result was recorded with the data for each individual dog.

The data were also compared with the coefficient of variation data for each echocardiographic parameter generated from the boxer repeatability study.

After detailed comparisons between serial scans for the Normal category, serial scans in the DCM, dFS, LVE and SAS categories were screened for differences of greater than 20% between initial and second (or final) scan.

B.19. *Comparison of right parasternal and left apical views for determining left ventricular volumes and left atrial areas*

The use of right parasternal long axis views is not a conventional method of determining left ventricular volume in human echocardiography, as the LV apex is not included. However, the LV is consistently longer with larger area from the right parasternal view in dogs compared with the left apical view. A study justifying the use of the RPS view is detailed in the Supplement (available from the author).

B.20. *Comparison of Simpson's rule and the single plane ellipse method in determining left ventricular volumes and ejection fraction*

A software upgrade was available during the end of this study which allowed the determination of LV volumes by Simpson's rule. A study was undertaken to attempt to investigate whether the two techniques were interchangeable, which is detailed in the Supplement (available from the author).

AN ECHOCARDIOGRAPHIC / DOPPLER EVALUATION OF A POPULATION OF NEWFOUNDLAND DOGS

RESULTS

B.21. *Population details*

A total of 223 scans were obtained from 165 individual Newfoundland dogs (69 males and 96 females). Fifty four dogs received a second scan and four dogs had a total of three scans.

B.21.1. *Body Weight*

Using data from 75 male dogs, the mean ± standard deviation body weight was 62.63 ± 9.2 kg, with a minimum weight of 43 kg and a maximum weight of 93 kg and a median weight of 63 kg. The data from 101 bitches gave a mean ± standard deviation body weight of 55.07 ± 5.6 kg, with minimum of 43 kg and maximum of 71 kg (Figure B.1.). Although the data was normally distributed, the equal variance test failed (assessed by SigmaStat v.2.0, SPSS inc.) and so the Mann-Whitney rank sum test was used to identify differences between the two groups. The male dogs were significantly heavier than the female dogs ($p<0.001$).

B.21.2. *Body Surface Area*

The mean ± standard deviation body surface area (BSA) for all Newfoundlands was 1.522 ± 0.143 m^2 (minimum, 1.24 m^2 and maximum, 2.08 m^2). The mean BSA for male Newfoundlands was 1.597 ± 0.155 m^2 (minimum, 1.24 m^2 and maximum, 2.08 m^2). The female Newfoundlands had a mean BSA of 1.465 ± 0.099 m^2 (minimum, 1.24 m^2 and maximum, 1.74 m^2). There was a significant difference between the sexes (Mann-Whitney rank sum test, $p<0.001$).

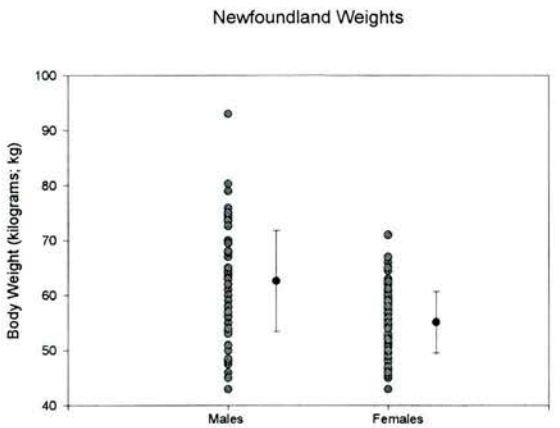


Figure B.1.

Newfoundland Weights. Raw data with means and standard deviations

B.21.3. *Raw data from the echocardiographic / Doppler examinations*

The raw data from all the Newfoundlands included in the study are not shown. However, the mean values for each parameter or calculated derivative are presented for all dogs with scans of diagnostic quality in the Supplement (available from the author). These include the reference given for each scan, numbered sequentially based on the videotape number, the individual identity by both DNA number (if DNA sample obtained) and P number (referring to the Pedigree/Draw data; see Section C). Dogs are grouped according to echocardiographic category (see later).

B.22. *Comparison between normal Newfoundlands and normal Newfoundlands under eight years old*

There was no significant difference for body weight, BSA or heart rate between the two groups, although age was significantly different ($p<0.001$) as it had been used to define the two groups.

Significant differences (unpaired two-tailed *t*-test or Mann-Whitney rank sum test) were identified for some of the two-dimensional echocardiographic parameters between the two groups. The left ventricular diastolic area and diastolic volumes were significantly smaller in the older group ($p<0.05$ for both) and the ejection

fraction was significantly lower in the older group (33.33% compared with 42.58%; $p<0.01$), with a smaller stroke volume in the older dogs ($p=0.001$). The only significant differences identified in the left atrial parameters was that LAls was smaller in the older dogs ($p<0.05$).

There were no significant differences between the two groups for any of the M-mode parameters. Consequently, the two groups were combined.

B.23. *The Normal Newfoundland group*

B.23.1. *Description of the Normal Newfoundland group*

The mean $\pm$ standard deviation age of the group (n=86) was 4.35 $\pm$ 2.57 years. It weighed 59.01 $\pm$ 2.14 kg, corresponding to a BSA of 1.53 $\pm$ 0.15 m^2. The mean heart rate during acquisition of two-dimensional echocardiographic parameters was 106.97 $\pm$ 16.32 beats per minute (bpm), corresponding to an average R-R interval of 0.575 $\pm$ 0.092 seconds, and during acquisition of right parasternal M-mode images was 104.12 $\pm$ 14.41 bpm, with R-R interval of 0.588 $\pm$ 0.081 seconds.

The results of descriptive statistical analysis of the two-dimensional and M-mode echocardiographic parameters are shown in Table B.4. Means, standard deviations, maximum and minimum data points, the 95% confidence interval of the mean with the lower and upper confidence limits are detailed. To include 95% of the normal population, the values of mean minus and plus two standard deviations are included, for evaluation of significant departures from the Normal group.

Table B.4.
Descriptive Statistics of the Normal Newfoundland population:
2D and M-mode parameters

PARAMETER	Mean	Standard deviation	Minimum	Maximum	95% Confidence interval of mean	Lower confidence limit	Upper confidence limit	Mean +2sd	Mean -2sd
2D parameters									
RPSLVad (sq.cm)	29.86	4.34	20.76	41.51	0.97	28.89	30.83	38.54	21.18
RPSLVld (mm)	79.80	6.10	63.52	92.25	1.36	78.44	81.16	92.00	67.60
RPSLVdv (mls)	95.56	22.14	56.60	159.13	4.93	90.63	100.49	139.84	51.28
RPSLVas (sq.cm)	20.73	4.90	13.62	53.87	1.09	19.64	21.82	30.53	10.93
RPSLVls (mm)	64.05	6.12	52.90	78.23	1.36	62.69	65.41	76.29	51.81
RPSLVsv (mls)	55.12	12.90	85.80	29.33	2.87	52.25	57.99	80.92	29.32
RPSEF (%)	41.77	8.26	15.17	58.50	1.84	39.93	43.61	58.29	25.25
RPSSV (mls)	40.74	13.54	11.40	77.13	3.01	37.73	43.75	67.82	13.66
EDVI (mls/sq.m)	62.39	13.24	39.45	97.45	3.45	58.94	65.84	88.87	35.91
ESVI (mls/sqm)	35.96	8.13	21.01	52.32	2.12	33.84	38.08	52.22	19.70
SVI (mls/sq.m)	26.60	8.15	11.99	26.79	2.12	24.48	28.72	42.90	10.30
RPSLAad (sq.mm)	1131.78	189.71	679.40	1718.72	41.68	1090.10	1173.46	1511.20	752.36
RPSLAld (mm)	36.73	3.63	29.20	50.50	0.80	35.93	37.53	43.99	29.47
RPSLAas (sq.mm)	1634.50	281.77	2542.02	1208.22	62.31	1572.19	1696.81	2198.04	1070.96
RPSLAls (mm)	41.29	3.98	54.28	33.25	0.88	40.41	42.17	49.25	33.33
LAEI	0.30	0.06	0.15	0.31	0.01	0.29	0.31	0.42	0.18
2DLAd (mm)	32.46	3.51	39.93	23.50	0.77	31.69	33.23	39.48	25.44
2DAod (mm)	29.56	2.42	36.08	25.10	0.53	29.03	30.09	34.40	24.72
2DLAd:Aod	1.10	0.11	0.81	1.39	0.02	1.08	1.12	1.32	0.88
M-mode parameters									
RVd (mm)	8.33	3.08	4.25	21.65	0.66	7.67	8.99	14.49	2.17
IVSd (mm)	10.66	1.13	8.02	13.77	0.24	10.42	10.90	12.92	8.40
LVIDd (mm)	45.35	4.03	35.78	54.85	0.86	44.49	46.21	53.41	37.29
LVpwd (mm)	10.28	1.13	8.10	13.18	0.24	10.04	10.52	12.54	8.02
IVSs (mm)	12.93	1.38	9.43	16.75	0.30	12.63	13.23	15.69	10.17
LVIDs (mm)	34.31	3.00	25.30	41.18	0.64	33.67	34.95	40.31	28.31
LVpws (mm)	13.69	1.38	11.18	16.45	0.30	13.39	13.99	16.45	10.93
%thIVS	22.97	10.25	-3.11	57.50	2.20	20.77	25.17	43.47	2.47
%thLVpw	34.45	10.00	8.83	55.33	2.14	32.31	36.59	54.45	14.45
FS(%)	24.47	3.21	20.00	34.83	0.69	23.78	25.16	30.89	18.05
EF(Teicholz)(%)	47.85	3.45	30.11	64.17	1.17	46.68	49.02	54.75	40.95
Mitral EPSS (mm)	5.69	1.68	2.80	10.16	0.36	5.33	6.05	9.05	2.33
LAs (mm)	24.13	4.06	14.37	35.68	0.87	23.26	25.00	32.25	16.01
Aod (mm)	29.18	2.71	23.72	37.50	0.58	28.60	29.76	34.60	23.76
LA:Ao	0.83	0.15	0.49	1.22	0.03	0.80	0.86	1.13	0.53
LVpwd/LVIDd	0.23	0.03	0.15	0.31	0.007	0.22	0.24	0.29	0.17
(LVpwd/BSA)/(LVIDd/BSA)	0.23	0.03	0.17	0.31	0.008	0.22	0.24	0.29	0.17
LVId/LVIDd	1.78	0.16	1.45	2.25	0.04	1.74	1.82	2.10	1.46
MAMseptal (mm)	11.29	2.08	7.55	20.64	0.45	10.84	11.74	15.45	7.13
MAMlateral (mm)	13.96	2.27	9.72	18.34	0.55	13.41	14.51	18.50	9.42
MAMmean (mm)	12.60	1.86	8.90	18.60	0.45	12.15	13.05	16.32	8.88
Indexed to BSA									
RVd/BSA (mm/sq.m)	5.40	1.87	2.80	12.97	0.46	4.94	5.86	9.14	1.66
IVSd/BSA (mm/sq.m)	6.97	0.86	4.86	9.18	0.21	6.76	7.18	8.69	5.25
LVIDd/BSA (mm/sq.m)	29.78	3.40	20.80	38.09	0.84	28.94	30.62	36.58	22.98
LVpwd/BSA (mm/sq.m)	6.72	0.74	5.29	8.58	0.18	6.54	6.90	8.20	5.24
IVSs/BSA (mm/sq.m)	8.57	1.48	5.99	16.99	0.37	8.20	8.94	11.53	5.61
LVIDs/BSA (mm/sq.m)	22.51	2.53	16.01	27.38	0.63	21.88	23.14	27.57	17.45
LVpws/BSA (mm/sq.m)	8.94	0.95	6.60	8.94	0.23	8.71	9.17	10.84	7.04
(LVId/LVIDd)/BSA	1.17	0.13	0.89	1.54	0.03	1.14	1.20	1.43	0.91
MAMseptal/BSA (mm/sq.m)	7.48	1.56	4.81	13.58	0.39	7.09	7.87	10.60	4.36
MAMlateral/BSA (mm/sq.m)	9.39	1.63	6.54	12.31	0.46	8.93	9.85	12.65	6.13

B.23.2. *Regression analyses for 2D and M-mode echocardiographic parameters in normal Newfoundlands*

The results of forward stepwise regression in identifying independent variables significantly influencing echocardiographic parameters are shown in Tables B.5. (M-mode parameters) and B.6. (2D parameters). The results of linear regression or multiple linear regression analyses are also shown in these tables, with equations given for both body weight and BSA where appropriate. The R value, R^2 and adjusted R^2 values and the level of statistical significance (p value) are also indicated. Linear regression graphs are shown in the Supplement (available from the author).

M-mode RVd was significantly influenced by weight or BSA. IVSd was related significantly to BSA but the relationship to body weight did not achieve statistical significance. IVSs was not significantly affected by any of the variables. LVpwd was influenced by body weight or BSA and the relationship was maintained in systole (LVpws). When indexed to BSA, no independent variable significantly affected RVd, IVSd, IVSs, LVpwd or LVpws. LVIDd was positively correlated to body weight or BSA but negatively to age. No parameter exerted a significant effect on LVIDs. However, when both LVIDd and LVIDs were indexed to BSA, significant but weak negative correlations were identified with age.

When the wall thickness was expressed as a fraction of the left ventricular internal diameter (LVpwd/LVIDd, relative wall thickness), no significant relationship was identified with any independent variable, although forward stepwise regression had indicated that age may be important.

No significant relationship was confirmed by linear regression analysis for FS% or the Teicholz derived ejection fraction (EFTeicholz). No independent variable influenced the percentage thickening of the IVS or the LVpw.

Table B.5. Linear regression and multiple linear regression equations for independent variables significantly influencing echocardiographic parameter: M-mode parameters

Echocardiographic parameter	Forward stepwise regression: Independent variables influencing parameter	Regression equation (BSA)	Regression equation (Wt)	R	R^2	Adjusted R^2	Significance
RVd	BSA (p=0.001), Wt (p=0.001)	RVd = -4.686 + (8.482 x BSA)		0.389	0.151	0.138	p=0.001
			RVd = -0.301 + (0.146 x Wt)	0.395	0.156	0.146	p=0.001
IVSd	BSA (p<0.048) NB Wt ns; p=0.052	IVSd = 7.671 + (1.924 x BSA)		0.246	0.0606	0.0457	p<0.05
			ns				
LVIDd	BSA (p=0.044) Wt (p=0.043), Age (p=0.004)	LVIDd = 37.718 + (6.585 x BSA) - (0.568 x age)		0.404	0.163	0.136	p<0.01
			LVIDd = 41.179 + (0.112 x Wt) - (0.566 x age)	0.404	0.164	0.137	p<0.01
LVIDd/BSA	Age (p=0.005)	LVIDd/BSA = 31.854 - (0.475 x age)		0.344	0.119	0.105	p<0.01
			ns				
LVpwd	BSA (p=0.002), Wt (p=0.002)	LVpwd = 5.977 + (2.783 x BSA)		0.379	0.144	0.13	p<0.01
			LVpwd = 7.479 + (0.0469 x Wt)	0.376	0.142	0.128	p<0.01
IVSs	No variables significantly predict parameter						
LVIDs	No variables significantly predict parameter						
LVIDs/BSA	Age (p=0.042)	LVIDs/BSA = 23.639 - (0.259 x age)		0.253	0.0642	0.0493	p<0.05
			ns				
LVpws	BSA (p=0.003); Weight (p=0.003)	LVpws = 8.561 + (3.312 x BSA)		0.367	0.135	0.121	p<0.01
			LVpws = 10.348 + (0.3558 x Wt)	0.365	0.133	0.119	p<0.01
FS	Age (p=0.031)	ns					ns
			ns				
EFTeicholz	Age (p=0.002)	ns					ns
			ns				
%thIVS	No variables significantly predict parameter						ns
%thLVpw	No variables significantly predict parameter						ns

Table B.5. Linear regression and multiple linear regression equations for independent variables significantly influencing echocardiographic parameter: M-mode parameters (continued)

Echocardiographic parameter	Forward stepwise regression: Independent variables influencing parameter	Regression equation (BSA)	Regression equation (Wt)	R	R^2	Adjusted R^2	Significance
EPSS	BSA(p<0.001), Wt (p<0.001)	EPSS = -1.466 + (4.684 x BSA)		0.402	0.162	0.148	p<0.001
			EPSS = 0.985 + (0.0802 x Wt)	0.405	0.164	0.151	p<0.001
EPSS/LVIDd	BSA (p=0.007), Wt (p=0.007)	EPSS/LVIDd = -0.00346 + (0.0846 x BSA)		0.332	0.110	0.096	p<0.01
			EPSS/LVIDd = 0.0412 + (0.00144 x Wt)	0.333	0.111	0.0969	p<0.01
LAs	BSA (p<0.001), Wt (p<0.001)	LAs = 6.415 + (11.378 x BSA)		0.398	0.158	0.145	p=0.001
			LAs = 12.275 + (0.196 x Wt)	0.404	0.163	0.15	p<0.001
Aod	BSA (p<0.001), Wt (p<0.001)	Aod = 15.344 + 9.131 x BSA)		0.504	0.254	0.242	p<0.001
			Aod = 20.082 + (0.157 x Wt)	0.51	0.26	0.249	p<0.001
LAs: Aod ratio	No variables significantly predict parameter						ns
LVpwd/LVIDd	Age (p<0.001)	ns					ns
2DRPSLVId:LVIDd ratio ("index of sphericity"	Sex (p=0.038)	RPSLVId:LVIDd = 1.91 - (0.0842 x sex)		0.258	0.0666	0.0547	p<0.05
			ns				
MAM (septal)	Sex (p = 0.015)	MAMsep = 13.4 - (1.369 x sex)		0.33	0.109	0.0979	p=0.002
			ns				
MAM(septal)/BSA	No variables significantly predict parameter						
MAM(lateral)	Age (p=0.002)	MAMlat = 15.162 - (0.288 x age)		0.315	0.099	0.0856	p=0.008
			ns				
MAM(lateral)/BSA	Age (p<0.001)	MAMlat = 10.692 - (0.304 x age)		0.472	0.223	0.207	p<0.001
			ns				
MAM (mean)	R-R (p=0.006); Age (p=0.002); Sex (p=0.002)	MAMmean = 19.465 - (5.864 x R-R) - (1.556 x sex-code) - (0.261 x age)	ns	0.537	0.288	0.260	p<0.001
		MAMmean = 13.155 - (0.168 x age)		0.236	0.0559	0.0443	p<0.05
		MAMmean = 14.262 - (1.195 x sex-code)		0.325	0.106	0.0948	p<0.01
		MAMmean vs R-R - non-significant p value					

Table B.6. Linear regression and multiple linear regression equations for independent variables significantly influencing echocardiographic parameter: 2D echo parameters

Echocardiographic parameter	Forward stepwise regression: Independent variables influencing parameter	Regression equation (BSA)	Regression equation (Wt)	R	R^2	Adjusted R^2	Significance
RPSLVId	BSA (p=0.035), Wt (p=0.035) & sex (p=0.008)	RPSLVId = 67.105 + (12.647 x BSA) - (4.242 x sex)		0.524	0.275	0.249	p<0.001
			RPS LV d = 73.563 + (0.219 x Wt) - (4.212 x sex)	0.524	0.274	0.248	p<0.001
RPSLVdv	BSA (p<0.001), Wt (p<0.001), & age (p<0.001)	RPSLVdv = -25.47 + (89.509 x BSA) - (3.642 x age)		0.609	0.371	0.0349	p<0.001
			RPSLVcv = 21.007 + (1.538 x Wt) - (3.626 x age)	0.608	0.37	0.347	p<0.001
EDVI	Age (p<0.001), R-R (p=0.018)	EDVI = 49.419 + (42.04 x R-R) - (2.53 x age)		0.515	0.264	0.238	p<0.001
RPSLVIs	BSA (p=0.011), sex (p<0.001) NB BSA = forced variable; multiple regression, p= 0.272 ns)	RPSLVIs = 63.927 + (6.461 x BSA) - (6.444 x sex)		0.578	0.334	0.31	p<0.001
			RPSLVIs = 67.211 + (0.112 x Wt) - (6.427 x sex)	0.578	0.334	0.31	p<0.001
RPSLVsv	BSA (p<0.001), Wt (p<0.001), age (p=0.028)	RPSLVsv = -17.916 + (51.975 x BSA) - (1.481 x age)		0.532	0.283	0.258	p<0.001
			RPSLVsv = 8.767 + (0.898 x Wt) - (1.474 x age)	0.533	0.285	0.259	p<0.001
ESVI	Sex (p=0.014), Age (0.021)	ESVI = 48.007 - (0.971 x age) - (5.047 x sex)		0.411	0.169	0.139	p<0.01
RPS EF	Sex (p=0.020)		ns				ns
RPS SV	Age (p<0.001), ESA (p=0.002), Wt (p=0.002)	RPSSV = -6.337 + (37.011 x BSA) - (2.204 x age)		0.525	0.276	0.25	p<0.001
			RPSSV = 13.097 + (0.632 x Wt) - (2.196 x age)	0.523	0.273	0.247	p<0.001
SVI	Age (p<0.001)	SVI = 32.775 - (1.436 x age)		0.428	0.183	0.169	p<0.001
RPSLAad	BSA (p<0.001), Wt (p<0.001) R-R (p=0.023)	RPSLAad = -234.508 + (689.644 x BSA)+(558.671 x R-R)		0.54	0.291	0.235	p<0.001
			RPSLAac = 123.004 + (11.87 x Wt) + (558.041 x R-R)	0.539	0.291	0.264	p<0.001
RPSLAId	BSA (p<0.001), Wt (p<0.001)	RPSLAId = 10.891 + (16.893 x BSA)		0.634	0.402	0.392	p<0.001
			RPSLAId = 19.8 + (0.288 x Wt)	0.638	0.407	0.396	p<0.001
RPSLAas	BSA (p<0.001), Wt (p<0.001)	RPSLAas = -275.646 + (1246.132 x BSA)		0.627	0.393	0.382	p<0.001
			RPSLAas = 381.61 + (21.242 x Wt)	0.63	0.397	0.387	p<0.001
RPSLAIs	BSA (p<0.001), Wt (p<0.001)	RPSLAIs = 15.074 + (17.232 x BSA)		0.6	0.36	0.349	p<0.001
			RPSLAIs = 24.168 + (0.294 x Wt)	0.603	0.364	0.353	p<0.001
LAEI ((LAas - LAad)/LAas)	R-R (p=0.038)	ns	ns				ns
RPSLAd	BSA (p=0.033) Wt (p=0.032)	RPSLAd = 22.446 + (6.637 x BSA)		0.277	0.0769	0.0615	p<0.05
			RPSLAd = 26.023 + (0.112 x Wt)	0.275	0.0758	0.0604	p<0.05
RPSAod	BSA (p<0.001)(Wt (p<0.001)	RPSAod = 14.981 = (9.654 x BSA)		0.558	0.311	0.3	p<0.001
			RPSAod = 20.109 + (0.164 x Wt)	0.558	0.312	0.3	p<0.001
2DLAd:Aod ratio	R-R (p=0.003)	2DLAd:aod = 0.876 + (0.395 x R-R)		0.338	0.115	0.103	p<0.01

Mitral EPSS significantly increased with body weight or BSA. Both M-mode LAs and Aod were significantly related to body weight and BSA. When the ratio of these two parameters (LAs:Aod) were considered, they were not influenced by any of the independent variables.

Mitral annulus motion, obtained from the septal annulus (MAMseptal), showed a significant relationship to gender, with males showing the greater MAMseptal. However, if MAMseptal was indexed to BSA, this was no longer evident, and no independent variable was identified by forward stepwise regression to significantly influence this parameter. Mitral annulus motion, obtained from the lateral annulus (MAMlateral), was significantly negatively influenced by age. This relationship was stronger and more significant after correcting for BSA. The mean mitral annulus motion (MAMmean) determined from these two values, was predicted by R-R interval, age and gender. A significant although weak relationship was shown between MAMmean and FS% ($R=0.247$; $p=0.041$), but not EF (determined by 2D techniques). MAMlateral also showed a similar relationship to FS ($R=0.239$; $p=0.048$), but the MAMseptal showed no significant relationship to these parameters of systolic function.

Attempts to quantify the degree of sphericity of the left ventricle, by considering the ratio of LV diastolic length, from the RPS view (LVld), and LVIDd showed that this ratio was significantly affected by sex. However, when this index was corrected for BSA, this variable was eliminated as being significantly associated with the value.

The diastolic and systolic LV lengths (LVld and LVls) were significantly influenced by BSA or weight and also gender; females had shorter left ventricles after correcting for BSA or weight in the multiple regression model. However, if LVld and LVls were indexed to BSA, sex was no longer a significant independent variable affecting the value. A new significant variable was identified affecting LVls/BSA, with R-R interval, by forward stepwise regression. This relationship was given by the linear regression equation:

LVIs/BSA = 33.704 + (14.681 x R-R)

($R=0.292$; $R^2= 0.0853$; adjusted $R^2 = 0.0687$; $p<0.05$)

The left ventricular diastolic volume (LVdv) was positively and significantly related to BSA or weight and negatively to age. When corrected for BSA, the left ventricular end diastolic volume index (EDVI), still showed the significant negative effect of age, and multiple regression indicated that the EDVI was also influenced positively by R-R interval. The left ventricular systolic volume (LVsv) is affected similarly by weight or BSA, and the left ventricular end-systolic volume index (ESVI) is significantly affected by age (lower in older dogs) and also sex (females have lower ESVI despite having corrected for BSA).

None of the independent variables showed a significant influence on EF derived by the single plane ellipse method. However, stroke volume (SV) was significantly affected by BSA or weight, and also significantly decreased with advancing age. When corrected for BSA, the stroke volume index (SVI) still showed a negative correlation for advancing age.

The left atrial parameters had also been obtained from an RPS long axis view. Left atrial area in diastole (LAad) was positively influenced by weight or BSA and R-R interval. The diastolic length of the left atrium, measured parallel to but slightly above the mitral annulus (LAld) also was positively correlated to BSA or weight. The left atrial systolic area (LAas) and length (LAls) were also significantly influenced by BSA or weight. When the left atrial emptying index (LAEI) was calculated, it was found that no independent variable was significantly related to this parameter.

The 2D short axis left atrial diastolic dimension (2D LAd) and the aortic diastolic dimension (2D Aod) both were positively related to BSA or weight. The 2D LAd:Aod ratio showed a significant positive relationship to the R-R interval.

B.23.3. *Regression analyses for Doppler echocardiographic parameters in Normal Newfoundlands*

The descriptive statistics for the normal Newfoundlands (older and younger than eight years old) are shown in Table B.7. These include the Doppler parameters for aortic outflow, mitral inflow, pulmonary venous flow and right sided Doppler velocities.

Tables B.8. to B.11. give the results of forward stepwise regression, multiple linear regression and linear regression, with R value, R^2 and adjusted R^2 and the level of significance (p value <0.05, <0.01 or <0.001). Linear regression graphs are included in the Supplement (available from the author).

Table B.7.
Descriptive Statistics of the Normal Newfoundland population:
Doppler parameters

PARAMETER	Mean	Standard deviation	Minimum	Maximum	95% Confidence interval of mean	Lower confidence limit	Upper confidence limit	Mean +2sd	Mean -2sd
Aortic Outflow parameters									
S/C Aov (m/s)	1.48	0.15	1.09	1.85	0.03	1.45	1.51	1.78	1.18
S/C Aovti (m)	0.17	0.02	0.07	0.21	0.00	0.17	0.17	0.20	0.14
S/C Ao dv/dt max (m/sq.s)	46.89	8.84	27.71	70.31	2.05	44.84	48.94	64.57	29.21
S/C Ao dv/dt mean (m/sq.s)	27.69	4.50	17.97	37.73	1.04	26.65	28.73	36.69	18.69
S/C LV PEP (corrected) (s)	0.072	0.010	0.052	0.093	0.002	0.070	0.074	0.092	0.052
S/C LV ET (s)	0.178	0.013	0.148	0.216	0.003	0.175	0.181	0.204	0.152
S/C Ao accel.time (s)	0.054	0.006	0.040	0.068	0.001	0.053	0.055	0.066	0.042
PEP:ET ratio (corrected)	0.403	0.057	0.275	0.543	0.013	0.390	0.416	0.517	0.289
VCf (circs/s)	1.38	0.20	0.96	1.89	0.04	1.34	1.42	1.78	0.98
PEP/√R-R	0.091	0.012	0.062	0.122	0.003	0.088	0.094	0.115	0.067
ET/√R-R	0.225	0.014	0.199	0.261	0.003	0.222	0.228	0.253	0.197
acc.t/√R-R	0.068	0.008	0.049	0.087	0.002	0.066	0.070	0.084	0.052
PEP:ET/√R-R	0.515	0.085	0.301	0.768	0.020	0.495	0.535	0.685	0.345
Vcf/√R-R	1.76	0.35	0.95	2.91	0.08	1.68	1.84	2.46	1.06
LAp. Aov (m/s)	1.17	0.14	0.92	1.54	0.03	1.14	1.20	1.45	0.89
Mitral inflow parameters									
MV Ev (m/s)	0.61	0.13	0.34	0.99	0.03	0.58	0.64	0.87	0.35
MV Av (m/s)	0.48	0.10	0.26	0.75	0.02	0.46	0.50	0.68	0.28
MV Evti (m)	0.063	0.011	0.040	0.100	0.002	0.061	0.065	0.085	0.041
MV Avti (m)	0.022	0.007	0.000	0.040	0.002	0.020	0.024	0.036	0.008
MV E duration (s)	0.192	0.022	0.119	0.234	0.005	0.187	0.197	0.236	0.148
MV E decel.time (s)	0.117	0.018	0.080	0.160	0.004	0.113	0.121	0.153	0.081
MV A duration (s)	0.090	0.012	0.053	0.126	0.003	0.087	0.093	0.114	0.066
MV E:A velocity ratio	1.32	0.40	0.83	3.19	0.087	1.23	1.41	2.12	0.52
MV E:A vti ratio	3.09	1.17	1.50	7.00	0.257	2.83	3.35	5.43	0.75
MV Ev/total vti (SV/s) (peak filling rate normalised forSV)	7.15	1.28	4.78	11.71	0.279	6.87	7.43	9.71	4.59
Isovolumic relaxation time									
IVRT (s)	0.062	0.010	0.046	0.060	0.002	0.060	0.064	0.082	0.042
Pulmonary venous flow parameters									
PVFArv (m/s)	0.24	0.11	0.12	0.73	0.024	0.22	0.26	0.46	0.02
PVFSv (m/s)	0.38	0.06	0.27	0.57	0.014	0.37	0.39	0.50	0.26
PVFDv (m/s)	0.37	0.05	0.27	0.54	0.012	0.36	0.38	0.47	0.27
PVFR2v (m/s)	0.16	0.05	0.09	0.33	0.011	0.15	0.17	0.26	0.06
PVFSvti (m)	0.063	0.012	0.040	0.090	0.003	0.060	0.066	0.087	0.039
PVFDvti (m)	0.082	0.020	0.040	0.130	0.005	0.077	0.087	0.122	0.042
PVF Ar duration (s)	0.067	0.013	0.000	0.094	0.003	0.064	0.070	0.093	0.041
PVF S duration (s)	0.248	0.032	0.080	0.305	0.007	0.241	0.255	0.312	0.184
PVF D duration (s)	0.339	0.060	0.194	0.484	0.014	0.325	0.353	0.459	0.219
PVF D deceleration (s)	0.199	0.050	0.097	0.348	0.011	0.188	0.210	0.299	0.099
PVF R2 duration (s)	0.062	0.009	0.048	0.079	0.005	0.057	0.067	0.080	0.044
Mitral A duration - PVF Ar duration	0.024	0.015	-0.007	0.075	0.003	0.021	0.027	0.054	-0.006
PVF S:D velocity ratio	1.04	0.19	0.73	1.74	0.044	1.00	1.08	1.42	0.66
PVF S:D vti ratio	0.81	0.26	0.40	1.50	0.06	0.75	0.87	1.33	0.29
PVF Svti/total forward vti	0.44	0.08	0.29	0.60	0.018	0.42	0.46	0.60	0.28
Right heart parameters									
RPS PAv (m/s)	0.72	0.12	0.42	0.97	0.0259	0.69	0.75	0.96	0.48
LPS PAv (m/s)	0.75	0.13	0.5	1.24	0.028	0.72	0.78	1.01	0.49
TVEv (m/s)	0.49	0.08	0.33	0.70	0.02	0.47	0.51	0.65	0.33
TVAv (m/s)	0.34	0.08	0.17	0.57	0.02	0.32	0.36	0.50	0.18
TV E:A velocity ratio	1.48	0.37	0.09	2.59	0.08	1.40	1.56	2.22	0.74

Table B.8. Linear regression and multiple linear regression equations for independent variables significantly influencing echo-Doppler parameters: Aortic outflow

Echocardiographic parameter	Forward stepwise regression: Independent variables influencing parameter	Regression equations	Linear regression equations	R	R^2	Adjusted R^2	Significance
S/C Aov	R-R (p= 0.004); Age (p=0.034)	S/C Aov = 1.818 - (0.0147 x age) - (0.0432 x R-R)		0.406	0.16	0.136	p<0.01
			S/C Acv = 1.543 - (0.0152 x age)	0.25	0.0624	0.0494	p<0.05
			S/C Acv = 1.751 - (0.419 x R-R)	0.321	0.103	0.0902	p<0.01
S/C Aovti	Age (p=0.03)	S/C Aovti = 0.18 - (0.00211 x age)		0.311	0.0969	0.0844	p<0.01
S/C Ao dv/dt max	R-R (p=0.003)	S/C Ao dv/dt max = 65.332 - (28.598 x R-R)		0.36	0.13	0.117	p<0.01
S/C Ao dv/dt mean	R-R (p=0.003)	S/C Ao dv/dt mean = 37.836 - (15.746 x R-R)		0.39	0.153	0.141	p<0.001
LV cPEP	Sex (p=0.003)	S/C LVcPEP = 0.0802 - (0.00545 x sexcode)		0.286	0.0815	0.07	p<0.01
			S/C LVcPEP = 0.059 + (0.0208 x R-R)	0.243	0.0591	0.0457	p<0.05
LVcPEP/√R-R	All variables eliminated from the model						
LV ET	R-R (p<0.001), Age (p=0.006)	S/C LV ET = 0.128 + (0.0858 x R-R) - (0.000907 x age)		0.739	0.545	0.532	p<0.001
			S/C LV ET = 0.123 + (0.0866 x R-R)	0.721	0.52	0.513	p<0.001
LV ET/√R-R	All variables eliminated from the model						
S/C Ao accel.time	Sex (p=0.003)	S/C Ao accel.time = 0.0617 - (0.00509 x sexcode)		0.438	0.192	0.181	p<0.001
S/C Ao acc.t/√R-R	Sex (p=0.030)	S/C Ao acc.t/√R-R = 0.0742 - (0.00409 x sex-code)		0.256	0.0656	0.0522	p<0.05
LV cPEP:ET ratio	Sex (p=0.024)	ns					ns
LV cPEP:ET/√R-R	All variables eliminated from the model						
Vcf	R-R (p<0.001)	Vcf = 1.903 - 0.818 x R-R)		0.435	0.189	0.177	p<0.001
Vcf/√R-R	All variables eliminated from the model						

Table B.9. Linear regression and multiple linear regression equations for independent variables significantly influencing echo-Doppler parameters: Mitral inflow

Echocardiographic parameter	Forward stepwise regression: Independent variables influencing parameter	Regression equations	Linear regression equations	R	R^2	Adjusted R^2	Significance
MV Ev	Age (p<0.001)	MV Ev = 0.0688 - (0.0189 x age)		0.374	0.14	0.129	p<0.001
MV Av	R-R (p<0.001); sex (p=0.029)	MV Av = 0.759 - (0.464 x R-R)	*(sex: p value not sig. in multiple linear regression)*	0.434	0.188	0.178	p<0.001
MV E:A velocity ratio	R-R (p=0.008)	MV E:A = 0.373 + (1.57 x R-R)		0.388	0.151	0.14	p<0.001
			MV E:A = 1.523 - (0.046 x age)	0.288	0.0828	0.0715	p<0.01
MV Evti	Age (p=0.004)	MV Evti = 0.069 - (0.00131 x age)		0.286	0.0819	0.0706	p<0.01
MV Avti	Weight (p=0.037) (and BSA p=0.037); sex (p=0.006)	MV Avti = -0.00118 + (0.000245 x Wt) + (0.00553 x sexcode)	*(p value ns for Wt, BSA or sexcode for lin.regressions)*	0.373	0.139	0.11	p<0.05
		MV Avti = -0.00869 + (0.0144 x BSA) + (0.0055 x sexcode)		0.373	0.139	0.11	p<0.05
MV E:A vti ratio	Age (p=0.044); sex (p=0.017)	MV Evti:Avti = 4.722 - (0.122 x age) - (0.712 x sexcode)		0.38	0.144	0.123	p<0.01
			MV Evti:Avti = 4.108 - (0.655 x sexcode)	0.279	0.078	0.0665	p<0.05
			MV Evti:Avti = 3.553 - (0.108 x age)	0.23	0.053	0.0412	p<0.05
MV Ev/total vti	Age (p=0.007)	MV Ev/total vti = 7.823 - (0.156 x age)		0.301	0.0905	0.0793	p<0.01
MV Av/total vti	R-R (p<0.001)	MV Av/total vti = 9.222 - (5.941 x R-R)		0.536	0.288	0.279	p<0.001
MV Evti/total vti	Age (p=0.014); Sex (p=0.029)	MV Evti/total vti = 0.835 - (0.00811 x age) - (0.0371 x sex-code)		0.388	0.151	0.13	p=0.001
			MV Evti/total vti = 0.774 - (0.00742 x age)	0.274	0.075	0.0636	p<0.05
			MV Evti/total vti = 0.794 - (0.0334 x sex-code)	0.249	0.0622	0.0506	p<0.05
MV Avti/total vti	Age (p=0.014); Sex (p=0.029)	MV Avti/total vti = 0.165 + (0.00811 x age) + (0.0371 x sex-code)		0.388	0.151	0.13	p=0.001
			MV Avti/total vti = 0.226 + (0.00742 x age)	0.274	0.075	0.0636	p<0.05
			MV Avti/total vti = 0.206 + (0.0334 x sex-code)	0.249	0.0622	0.0506	p<0.05
MV E duration	R-R (p=0.001);Age (p=0.013)	MV Edur = 0.149 + (0.0559 x R-R) + (0.00217 x age)		0.313	0.0977	0.0745	p<0.05
MV Edur/$\sqrt{R-R}$	Age (p=0.006)	MV Edur/$\sqrt{R-R}$ = 0.234 + (0.0034 x age)		0.277	0.0765	0.0648	p<0.05
MV Edecel.time	E dur (p<0.001) if included as an independent variable	MVEdecel = -0.00786 + (0.641 x Edur)		0.726	0.527	0.521	p<0.001
	R-R (p<0.001); Age (p=0.004) if Edur excluded	MV Edecel = 0.067 + (0.0617 x R-R) + (0.00278 x age)		0.474	0.225	0.204	p<0.001
			MV E decel = 0.0861 + (0.05 x R-R)	0.272	0.0741	0.0621	p<0.05
			MV Edecel = 0.106 + (0.00239 x age)	0.336	0.113	0.101	p<0.01
MV Edecel/$\sqrt{R-R}$	Age (p=0.002)	MV E decel/$\sqrt{R-R}$ = 0.134 + (0.00374 x age)		0.424	0.18	0.169	p<0.001
MV A duration	R-R (p<0.001)	MV Adur = 0.0638 + (0.0431 x R-R)		0.341	0.116	0.105	p<0.01
MV A dur/$\sqrt{R-R}$	All variables eliminated from the model						
Isovolumic relaxation time							
IVRT	R-R (p=0.04)	IVRT = 0.488 + (0.0212 x R-R)		0.239	0.0572	0.0449	p<0.05
IVRT/$\sqrt{R-R}$	All variables eliminated from the model						

Table B.10. Linear regression and multiple linear regression equations for independent variables significantly influencing echo-Doppler parameters
Pulmonary venous flow (PVF)

Echocardiographic parameter	Forward stepwise regression: Independent variables influencing parameter	Regression equations	Linear regression equations	R	R^2	Adjusted R^2	Significance
PVF Arv	R-R (p<0.001); age (p=0.043)	PVF Arv = 0.584 - (0.54 x R-R)	(age not sig. p value in multiple linear regression)	0.505	0.255	0.245	p<0.001
			PVF Arv = 0.187 + (0.0121 x age)	0.262	0.0685	0.0555	p<0.05
PVF Sv	R-R (p<0.001); age (p=0.004)	PVF Sv = 0.514 - (0.266 x R-R) + (0.00992 x age)		0.609	0.371	0.353	p<0.001
			PVF Sv = 0.585 - (0.314 x R-R)	0.5	0.25	0.239	p<0.001
			PVF Sv = 0.336 + (0.0114 x age)	0.419	0.175	0.164	p<0.001
PVF Dv	No independent variable significantly predicts PVF Dv						ns
PVF R2v	R-R (p=0.002)	PVF R2v = 0.271 - (0.172 x R-R)		0.37	0.137	0.125	p=0.001
PVF Svti	Age (p=0.011)	PVF Svti = 0.0558 + (0.00163 x age)		0.314	0.0985	0.086	p<0.01
PVF Dvti	R-R (p<0.001); sex (p=0.013)	PVF Dvti = 0.0111 + (0.111 x R-R)		0.554	0.306	0.296	p<0.001
PVF S:D velocity ratio	R-R (p=0.019); age (p=0.014); sex (p=0.045)	PVF S:D = 1.328 - (0.59 x R-R) + (0.0216 x age)	*(Sexcode not sig. p value in multiple linear regression)*	0.439	0.193	0.169	p<0.001
			PVF S:D = 1.481 - (0.694 x R-R)	0.362	0.131	0.118	p<0.01
			PVFS:D = 0.930 + (0.0262 x age)	0.315	0.099	0.0865	p<0.01
PVF S:D vti ratio	R-R (p<0.001) Age (p<0.001)	PVF Svti:Dvti = 1.453 - (1.241 x R-R) + (0.373 x age)		0.625	0.391	0.373	p<0.001
			PVF Svti:Dvti = 1.719 - (1.419 x R-R)	0.541	0.292	0.282	p<0.001
			PVF Svti:Dvti = 0.618 + (0.0466 x age)	0.409	0.167	0.156	p<0.001
PVF S/total forward vti	R-R (p<0.001) Age (p=0.05)	PVF S/tot = 0.640 - (0.384 x R-R) + (0.0106 x age)		0.635	0.403	0.386	p<0.001
			PVF S/tot = 0.715 - (0.435 x R-R)	0.559	0.313	0.303	p<0.001
			PVF S/tot = 0.381 + (0.0136 x age)	0.403	0.163	0.151	p<0.001
PVF A duration	R-R (p=0.02)	ns					ns
PVF S duration	R-R (p=0.01)	PVF Sdur = 0.175 + (0.113 x R-R)		0.351	0.123	0.11	p<0.01
PVF D duration	R-R (p<0.001); age (p=0.03)	PVF Ddur = 0.0649 + (0.455 x R-R) - (0.00423 x age)		0.81	0.656	0.646	p<0.001
			PVF Ddur = 0.0348 + (0.476 x R-R)	0.795	0.632	0.626	p<0.001
			PVF Ddur = 0.373 - (0.00841 x age)	0.318	0.101	0.0886	p<0.01
PVF D deceleration time	Ddur (p<0.001) if included as an independent variable	PVF Ddecel = -0.00241 + (0.594 x Ddur)		0.728	0.529	0.523	p<0.001
	R-R (p<0.001) if Ddur excluded	PVF Ddecel = 0.00328 + (0.306 x R-R)		0.62	0.385	0.376	p<0.001
PVF R2 duration	No independent variable significantly predicts PVF R2v	ns					ns
Mitral Adur. - PVF Ar dur	No independent variable predicts the difference between A-Ar duration						ns
PVF S dur vs LAEI		PVF Sdur = 0.285 - (0.123 x LAEI)		0.247	0.0608	0.0472	p<0.05
PVF Svti:Dvti vs LAEI		PVF Svti:Dvti = 1.015 - (0.957 x LAEI)		0.235	0.0553	0.0416	p<0.05

Table B.11. Linear regression and multiple linear regression equations for independent variables significantly influencing echo-Doppler parameters: Right heart (pulmonary artery and tricuspid valve)

Echocardiographic parameter	Forward stepwise regression: Independent variables influencing parameter	Regression equations	Linear regression equations	R	R^2	Adjusted R^2	Significance
RPS PAv	Age (p=0.003), R-R (p=0.015)	RPSPAv = 0.92 - (0.221 x R-R) - (0.0141 x age)		0.351	0.123	0.1	p<0.01
			RPS PAv = 0.775 - (0.0131 x age)	0.278	0.077	0.065	p<0.05
LPS PAv	Age (p<0.001), R-R (p<0.001)	LPSPAv = 1.014 - (0.313 x R-R) - (0.0146 x age)		0.37	0.137	0.14	p<0.01
			LPSPAv = 0.909 - (0.251 x R-R)	0.22	0.0486	0.0365	p<0.05
			LPSPAv = 0.811 - (0.0134 x age)	0.265	0.0702	0.059	p<0.05
TV Ev	Age (p= 0.008)	TVEv = 0.542 - (0.0126 x age)		0.359	0.129	0.118	p<0.001
TV Av	R-R (p=0.001)	TVAv = 0.545 - (0.318 x R-R)		0.441	0.195	0.185	p<0.001
TV E:A velocity ratio	Age (p=0.028), R-R (p=0.012)	TV E:A = 0.885 + (0.194 x R-R) - (0.0371 x age)		0.464	0.215	0.195	p<0.001
			TV E:A = 0.65 + (1.315 x R-R)	0.292	0.0855	0.0742	p<0.01
			TV E:A = 1.673 - (0.0445 x age)	0.397	0.158	0.147	p<0.001

B.23.3.1. *Aortic flow*

Table B.8. gives the details for the Doppler parameters of aortic outflow. The subcostal view was used for the detailed analysis, although the velocity recorded from the left apical view is noted, and systolic time intervals determined from the left apical view were used if an inadequate subcostal signal was obtained, as there is no significant difference between the two views for time intervals (data not shown). Technically adequate subcostal signals were obtained in 74 out of 86 normal dogs (86%).

Aov showed a trend to lower values with a longer R-R interval and significantly declined with age. The aortic vti tended to decline with age. There was no association between age and R-R interval identified. Both peak and mean aortic acceleration (dv/dt_{max} and dv/dt_{mean}) declined with R-R interval. ET showed a significant relationship with mean R-R interval, but the influence of heart rate was less strong and less significant on PEP. Male dogs appeared to have a significantly longer PEP than bitches, although this relationship was not present after indexing PEP to the square root of the R-R interval. Males also showed a trend to faster acceleration time for aortic flow, a relationship which was still apparent although less significant after normalisation to the √R-R. No independent variable was a significant predictor of the PEP:ET ratio. Vcf was lower with longer R-R. No significant variables predicted parameters normalised for heart rate (PEP/√R-R, ET/√R-R, PEP:ET/√R-R or Vcf/√R-R).

B.23.3.2. *Mitral inflow*

Table B.9 shows the details about the analysis of mitral inflow in this normal Newfoundland population. Mitral Ev, the E:A velocity ratio, Evti and E:A vti ratio were all inversely associated with advancing age. E duration and E deceleration time increased with advancing age. The mitral peak filling rate, normalised to mitral stroke volume, declined with advancing age. The mitral A wave did not appear to be influenced by age, but increasing R-R interval was associated with lower Av. This relationship with R-R interval was retained after normalising Av to total mitral vti.

Forward stepwise regression had indicated that gender may influence A wave velocity, although this was not statistically significant. However, the Avti was influenced by both body weight and gender (female dogs tended to have higher A wave vti). On their own, in simple linear regression models, however, neither weight or BSA nor gender resulted in a statistically significant p value.

The proportion of early left ventricular filling (Evti/total vti) was influenced by both age and gender in a multiple linear regression model. This proportion declined with advancing age and was significantly lower in bitches. Similar but opposing relationships were evident for the proportion of late ventricular filling (Avti/total vti) and age and gender, with the larger ratio in older dogs and males.

E duration was influenced by R-R interval as well as age. The influence of age was still significant, increasing in older dogs, after E duration was indexed to √R-R. The major predictor of E deceleration time was mitral E duration. However, if E duration was eliminated as an independent variable, the major effects on E deceleration time were R-R interval as well as age. E deceleration/√R-R interval significantly increased with advancing age. The R-R interval also influenced A duration and isovolumic relaxation time (IVRT). Neither Adur/√R-R or IVRT/√R-R were associated with any other independent variable.

B.23.3.3. *Pulmonary venous flow*

Table B.10. details the analysis of the PVF parameters. Arv, Sv, Svti, S:D velocity ratio and S:D vti ratio all increased with advancing age. The systolic fraction of total forward flow also increased with age. The S:D velocity ratio, S:D vti ratio and systolic fraction of total forward flow were also influenced by R-R interval, decreasing at slower heart rates. No independent variable influenced Dv. R2v increased with longer R-R intervals. S and D durations were both associated with R-R interval. The major predictor of D deceleration time was D duration, although if D duration was not included as an independent variable, the major effect on D deceleration time was R-R interval. D duration also showed a weak although

significant inverse association with age. No independent variable significantly predicted Ar or R2 durations. No independent variable was shown to influence any PVF wave duration indexed to √R-R interval.

The normality plot of linear regression residuals and PVF velocities was not perfectly linear, but attempts at simple transformation of the independent variables, such as the R-R interval under test did not improve linearity. Arv was most effected, showing non-normal distribution when regressed to both R-R interval (Figure B.2.) and age. It is possible that the velocities should have been transformed. The S and D velocity residuals were relatively normally distributed; the major effects were seen with Ar and R2 velocities.

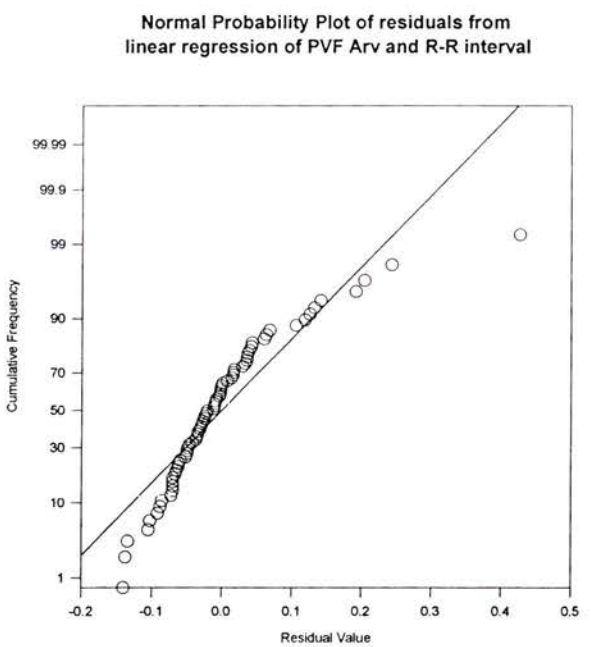


Figure B.2.
Plot of residuals from linear regression of PVF Arv and R-R interval is not consistent with normal distribution

Mitral A duration was longer than the PVF Ar duration in all but three dogs, when the difference ranged from 0.002 - 0.007 seconds, which is less than Doppler

temporal resolution (10 milliseconds), so is not significant. The mean difference in durations was 0.024 seconds and no independent variable influenced this difference, which is consistent with the data from man (Klein *et al* 1998).

The various parameters of PVF were assessed by forward stepwise regression for any association with the LAEI. The left atrial emptying index proposed by Clarkson and others (1995) was an independent negative predictor of S wave duration and S:D vti ratio. Increase in the LAEI was associated with decreased S duration and decreased S:D ratio.

B.23.3.4. *Right heart*

Table B.11. details the findings from the limited analysis of right sided function. PAv was almost always higher from the LPS view (mean for group; 0.75 m/s) than the RPS view (mean for group; 0.72 m/s). In general, the spectral signal was better from the LPS view, although in some dogs lack of perfect alignment resulted in a bifid tipped appearance to the envelopes. PAv was lower for longer R-R intervals and also for increasing age. Tricuspid Ev and the E:A velocity ratio tended to decrease with advancing age. Lengthening R-R interval was associated with lower Av and higher E:A velocity ratio.

B.23.4. *Summary of the influence of the independent variables on echo-Doppler parameters in Normal Newfoundlands*

The influence of the independent variables, body size, gender, age or mean R-R interval on specific echo-Doppler indices are summarised in the table displayed in Appendix B.1. (Volume II).

B.23.5. *Inter-relationship of certain echo-Doppler parameters*

A matrix shown in Appendix B.2. (Volume II) displays significant correlation coefficients between certain echo-Doppler parameters assessing left ventricular function.

B.24. *Comparison of the Newfoundland categories*

B.24.1. *Recorded details and summary of groups*

The details for each category of Newfoundlands are not included, although are available in the Supplement (available from the author).

There were 86 dogs in the Normal group (40 males, 46 females), 35 dogs in the DCM group (13 males, 22 females), 29 dogs in the depressed fractional shortening < 18% (dFS<18%) group (11 males, 18 females), 24 dogs in the dFS18-20% group (nine males and fifteen females), eight dogs in the left ventricular enlargement (LVE) group (two males and six females) and a total of forty dogs in the subaortic stenosis (SAS) group (20 males and 20 females). Compared with the total population (69 males and 96 females), there was no significant sex predisposition identified for any group (χ^2 test; $p>0.05$). A significant difference in age was identified between the groups (ANOVA on ranks, $p<0.001$). Dunn's method of all pair-wise multiple comparison procedure identified that the DCM group were significantly older that the Normal dogs, the SAS group and the dFS18-20% group ($p<0.05$). Although the SAS group showed a trend to lower weight and BSA, this was not statistically significant (ANOVA). There was no significant difference in the heart rate recorded during imaging of the RPS long-axis view between any of the groups, although the LVE group tended to have a slower rate than the other groups (ANOVA). The general comparisons between groups are illustrated in Table B.12.

Table B.12.
Means and Standard deviations for Newfoundland groups with One Way Analysis of Variance to identify differences between groups: 2D echocardiographic parameters

	NORMAL Group		DCM group		dFS<18% group		dFS18-20 group		LVE group		SAS group		ANOVA or ANOVA on RANKS	Multiple Pairwise comparisons: (TUKEY test or DUNNS method)
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd		
General														
Age	4.35	2.57	7.69	2.43	5.71	2.02	4.23	2.20	5.90	3.12	3.39	2.27	p<0.001	DCM>SAS, dFS18-20% & Normal groups
Weight	59.01	8.61	59.85	7.89	59.47	9.71	58.89	6.95	58.40	6.26	54.96	7.52	ns	
BSA	1.53	0.15	1.55	0.14	1.54	0.17	1.53	0.12	1.52	0.11	1.46	0.13	ns	
HR	106.97	16.32	107.62	16.32	105.33	16.85	107.59	21.27	94.13	15.81	113.77	20.88	ns	
2D LV parameters														
RPSLVad	29.86	4.34	34.35	5.61	29.12	4.97	30.47	4.13	31.50	4.17	31.78	4.35	p<0.001	DCM>dFS<18% & Normal groups
RPSLVid	79.80	6.10	82.13	6.90	79.35	6.43	80.54	6.17	78.96	5.02	80.86	5.51	ns	
RPSLVdv	95.56	22.14	123.16	34.34	91.05	25.79	97.89	20.64	107.78	21.09	107.12	23.81	p<0.001	DCM>dFS<18% & Normal groups
RPSLVas	20.73	4.90	26.72	5.61	21.08	4.19	21.00	3.03	20.62	3.53	21.37	3.54	p<0.001	DCM>Normal & dFS18-20% groups
RPSLVis	64.05	6.12	70.30	7.61	65.71	6.30	65.33	5.29	61.54	7.43	65.09	5.91	p<0.001	DCM>LVE, Normal & SAS groups
RPSLVsv	55.12	12.90	88.11	30.40	57.87	18.63	57.91	14.17	58.67	14.06	59.81	15.39	p<0.001	DCM>Normal, dFS<18%, dFS18-20% & SAS groups
RPSEF	44.77	8.26	28.93	10.04	35.67	12.52	39.67	9.99	45.31	9.28	44.06	8.33	p<0.001	DCM< LVE, SAS, Normal & dFS18-20% groups
RPSSV	40.74	13.54	36.28	14.53	32.87	14.43	40.08	13.52	49.10	14.97	47.66	13.81	p<0.001	LVE>dFS<18% & SAS>dFS<18%
EDVI	62.39	13.24	76.72	18.25	56.99	11.96	63.63	13.01	71.43	16.73	72.79	13.64	p<0.001	DCM > dFS<18%, Normal & dFS18-20% groups & SAS > Normal & dFS<18% groups
ESVI	35.96	8.13	55.17	17.00	35.35	9.41	37.54	8.17	38.30	9.52	40.04	9.19	p<0.001	DCM>LVE, dFS<18%, Normal, dFS18-20% & SAS groups
SVI	26.60	8.14	22.14	9.00	21.39	8.71	26.14	9.47	33.14	11.76	32.78	8.42	p<0.001	LVE>dFS<18% & SAS>dFS<18%
R-R	0.58	0.09	0.57	0.08	0.58	0.09	0.58	0.11	0.65	0.10	0.55	0.10	ns	
SVI/sq.rt.R-R	35.68	10.60	29.60	12.50	27.92	11.42	34.37	11.03	42.00	14.32	44.87	11.77	p=0.01	SAS> dFS<18%, DCM, dFS18-20% & Normal
2D LA parameters														
RPSLAad	1131.78	189.71	1431.95	531.44	1088.57	217.26	1181.88	309.70	1160.56	254.20	1144.53	212.85	p<0.001	DCM > dFS<18%, Normal, SAS & dFS18-20%
RPSLAid	36.73	3.63	41.06	5.44	36.12	3.18	37.45	4.24	37.70	4.18	36.69	3.41	p<0.001	DCM > dFS<18%, SAS, Normal, dFS18-20%
RPSLAas	1634.50	281.77	1899.49	599.86	1509.98	292.21	1633.52	337.01	1582.61	378.01	1661.23	306.96	p=0.01	DCM > dFS<18%
RPSLAis	41.29	3.98	45.58	5.72	40.86	3.36	42.33	4.20	41.57	4.35	41.68	4.00	p<0.001	DCM > dFS<18% & Normal
LAEI	0.30	0.06	0.25	0.10	0.28	0.06	0.28	0.07	0.26	0.07	0.31	0.08	p<0.01	DCM < SAS, Normal
RPSLAd	32.46	3.51	35.77	5.21	30.42	3.31	33.39	4.29	33.08	4.63	32.63	3.14	p<0.001	DCM > dFS<18%
RPSAod	29.56	2.42	30.80	3.41	30.32	3.01	29.49	2.62	28.09	1.85	28.89	2.11	p<0.05	No significant pairwise differences identified
2DLAd:Aod	1.10	0.11	1.17	0.18	1.01	0.11	1.13	0.12	1.17	0.11	1.13	0.12	p<0.01	dFS<18% < LVE, DCM, dFS18-20%, SAS and Normal
R-R	0.58	0.09	0.57	0.08	0.58	0.09	0.58	0.11	0.65	0.10	0.55	0.10	ns	
LA parameters / BSA														
LAad/BSA	744.04	109.16	956.82	355.28	701.38	112.39	786.58	184.21	774.67	227.81	787.40	127.88	p<0.01	DCM > dFS<18% & Normal
LAid/BSA	24.05	2.11	26.89	3.92	23.60	2.38	24.68	2.64	24.89	4.18	25.24	2.84	p<0.05	DCM > dFS<18% & Normal
LAas/BSA	1065.36	151.35	1252.91	381.90	969.30	154.74	1087.26	211.74	1050.33	344.60	1137.92	176.40	p<0.01	DCM & SAS > dFS<18%
LAis/BSA	27.14	2.48	29.55	3.60	26.52	2.88	28.07	2.84	27.57	4.76	28.67	2.76	p<0.01	DCM > dFS<18% and Normal
2DLAd/BSA	21.42	2.60	23.77	3.77	19.68	2.05	21.58	2.54	22.23	4.79	22.61	2.50	p<0.001	DCM & SAS > dFS<18%
2DAod/BSA	19.52	1.65	19.76	1.82	19.84	2.89	19.46	1.73	18.66	2.44	19.87	2.10	ns	

B.24.2. *2D echocardiographic parameters (Left ventricle, LV)*

The mean and standard deviation for each parameter from the dogs in each Newfoundland group are listed in Table B.12. The results from One Way analysis of Variance (ANOVA) or the Kruskall-Wallis One Way Analysis of Variance on ranks are also shown in this table, with multiple pair-wise comparisons performed by the Tukey test or Dunn's method. Corresponding box and whisker plots illustrating the differences between groups are shown in the Supplement (available from the author).

LVdv was significantly greater in the DCM group than the Normal group or the dFS<18% group. The same significant differences were also identified for the left ventricular diastolic area (LVad) although no significant differences were identified from the left ventricular length (LVld), despite a trend to a longer ventricle in the DCM group. The DCM group had a significantly larger EDVI than Normal, dFS<18% and dFS18-20% groups and the SAS group had a larger EDVI than the Normal and dFS<18% groups.

LVsv was significantly larger in the DCM group than the Normal, dFS<18%, dFS18-20% and the SAS groups. The DCM group had a larger LVas than the normal or dFS<18% groups, and a longer LVls than the LVE, Normal and SAS groups. ESVI was significantly larger in the DCM group was than all other groups.

The EF was significantly lower in the DCM group than the LVE, SAS, Normal and dFS18-20% groups. The LVE group tended to have a higher EF than the Normal group and SAS group although this was not statistically significant. Calculation of the total left ventricular stroke volume (SV) from the single plane ellipse method showed a larger SV for the LVE group, although this was only significantly different from the dFS<18% group. The SAS group also showed a higher SV than the dFS<18% group. These differences were still evident if SV was indexed to BSA (the stroke volume index, SVI). Although there was no significant difference between the heart rates in the various categories, the LVE group had a slightly slower heart rate and the SAS group a slightly higher HR than the other groups. The SVI was

normalised to the square root of the R-R interval to allow further comparisons between the groups. Although the LVE group still tended to have a higher value than the other groups, this was no longer statistically significant, but the SAS group showed a significantly larger SVI/√R-R than the dFS<18%, DCM, dFS18-20% and the Normal groups.

B.24.3. *2D echocardiographic parameters (Left atrium, LA)*

The means, standard deviations and the results of ANOVA and multiple pair-wise comparisons are shown in Table B.12.

There was no significant difference between the R-R intervals for any of the groups. Left atrial diastolic area (LAad) and length (LAld) were significantly greater in the DCM group than the other five groups, although once indexed to BSA, the differences were only significant for the Normal and the dFS<18% groups, since the dFS18-20%, the LVE and the SAS groups showed a trend to larger left atria. The only significant difference in LAas was between the DCM and the dFS <18% groups. Once normalised to BSA, the SAS group also showed a larger LAas/√R-R than the dFS<18% group. The DCM LAls was significantly greater than the dFS<18% and the Normal groups, which was also evident after indexing to BSA, although this figure showed a trend to larger LAas/√R-R in the LVE and the SAS groups.

The left atrial emptying index (LAEI), showed lower values in the DCM group and the LVE group (0.25 and 0.26 respectively) than the Normal and SAS groups (0.30 and 0.31 respectively), although this was only significant for the DCM group. The two dFS groups were intermediate, both at 0.28.

The 2D LAd showed a significantly larger DCM measurement than dFS<18%. Once indexed to BSA, this dimension was significantly larger for both the DCM and SAS groups compared with the dFS<18% group. The trend to larger LAd in the LVE group did not achieve statistical significance. 2D Aod did show a significant ANOVA result ($p<0.05$), although no significant pair- wise differences were

identified, and ANOVA showed no significant differences between Newfoundland groups for Aod once the parameter was indexed to BSA. 2D LAd:Aod showed a smaller ratio for dFS<18% than all other groups.

B.24.4.1. *Presence of mitral regurgitation*

A number of dogs in each group were shown to have mitral regurgitation (MR) based on CFDE, which was scored as previously described (B.15.1). Results are presented in Table B.13.

Table B.13.
Newfoundlands in different categories with mitral regurgitation grades

Newfoundland Group	0	0.5	1	2	3	4	TOTAL
Normal	45 (52.3%)	14 (16.3%)	26 (30.2%)	1 (1.2%)	0	0	86
DCM	8 (22.9%)	2 (5.7%)	12 (34.3%)	10 (28.6%)	3 (8.5%)	0	35
dFS<18%	14 (48.3%)	2 (6.9%)	12 (41.4%)	1 (3.4%)	0	0	29
dFS18-20%	12 (50%)	3 (12.5%)	8 (33.3%)	1 (4.2%)	0	0	24
LVE	2 (25%)	2 (25%)	2 (25%)	2 (25%)	0	0	8
SAS	19 (47.5%)	4 (10%)	15 (37.5%)	2 (5%)	0	0	40

χ^2 analysis showed that there was significantly more MR in the DCM group than the normal group ($p<0.001$). There was no significant difference between Normal Newfoundlands or the Newfoundlands in other categories when the MR severity was considered.

Forward stepwise regression failed to identify any relationship between the grade of MR and the parameters LAd, LAld, LAas, LAls, LAEI, 2DLAd or 2DLAd:Aod ratio for Newfoundlands in the Normal, dFS<18%, dFS18-20%, LVE or SAS categories. However, in the DCM group, a positive relationship was identified

between MR grade and LAld, LAas and LAls (all $p<0.001$) and 2DLAd:Aod ($p=0.017$). The linear regression equations for LAas and LAls are shown in Table B.24.

B.24.4.2. *Calculation of left ventricular dP/dt from mitral regurgitant jet*

Good quality CW spectral Doppler recordings of mitral regurgitant jets were obtained from only five scans from four different individual dogs which were of sufficient diagnostic quality to allow the LV dP/dt to be calculated. Four of the scans were in the DCM category, and one was in the dFS<18% category. LV dP/dt was determined as previously described (B.10.4.4.2.). The following results were obtained (Table B.14.). Mean and standard deviations were shown for pressures (pr.) determined by the modified Bernouille equation.

Table B.14.
Calculation of LV dP/dt from mitral regurgitant jets

Individual	Scan	Category	MR pr.1 (mmHg)	MR pr.2 (mmHg)	dP (mmHg)	dt (seconds)	LV dp/dt (mmHg/s)
- /P706	NF5/1	dFS<18%	4.00 ± 0.00	58.76 ± 12.61	54.76 ± 12.61	0.04 ± 0.00	1370.16 ± 245.44
NF002/P500	NF5/6	DCM	4.00 ± 0.01	64.80 ± 0.80	60.80 ± 0.80	0.04 ± 0.00	1375.08 ± 45.23
NF017/P543	NF7/1	DCM	4.00	64.4	60.4	0.026	2323.08
NF042/P542	NF9/5	DCM	4.00 ± 0.00	64.40 ± 2.77	60.40 ± 2.77	0.02 ± 0.01	3743.89 ± 1410.19
NF002/P500	NF12/8	DCM	4.26 ± 0.65	62.82 ± 2.95	58.56 ± 2.48	0.04 ± 0.01	1434.78 ± 335.95

The mean ± standard deviation dP/dt for this limited group of individuals is 2049.40 ± 1029.57 mmHg/s, with a range from a minimum of 1370.16 to a maximum of 3743.89 mmHg/s.

B.24.5. *M-mode parameters*

Means and standard deviations for each M-mode parameter and the six different Newfoundland groups are given in Table B.15. Differences between groups are identified by ANOVA or ANOVA on ranks, with the multiple pair-wise comparisons (Tukey test or Dunn's method). Box and whisker plots are displayed in the Supplement (available from the author).

Table B.15.
Means and Standard deviations for Newfoundland groups
with One Way Analysis of Variance to identify differences between groups:
M-mode echocardiographic parameters

	NORMAL Group		DCM group		dFS<18% group		dFS18-20 group		LVE group		SAS group		ANOVA or ANOVA on RANKS	Multiple Pairwise comparisons: (TUKEY test or DUNNS method)
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd		
M-mode parameters														
RVd	8.33	3.08	8.79	2.99	7.73	2.76	8.03	2.38	8.33	2.22	8.11	3.19	ns	
IVSd	10.66	1.13	11.02	1.56	10.57	1.30	10.30	1.25	11.43	1.71	11.20	1.62	ns	
LVIDd	45.35	4.03	51.14	5.63	42.88	4.56	43.85	3.69	54.70	3.42	47.60	4.38	p<0.001	LVE > dFS<18%, dFS18-20%, Normal & SAS. DCM > dFS<18%, dFS18-20%, Normal & SAS. SAS > dFS<18% & dFS18-20%
LVpwd	10.28	1.13	10.29	1.23	9.75	0.67	10.08	1.40	10.44	1.12	10.49	1.23	ns	
IVSs	12.93	1.38	12.81	1.46	11.91	1.50	11.95	1.33	14.57	2.23	13.29	1.95	p<0.001	LVE > dFS<18%, dFS18-20%, DCM & Normal. SAS > dFS<18%, dFS18-20%. Normal > dFS<18%
LVIDs	34.31	3.00	42.66	4.97	36.79	3.62	35.43	2.90	40.73	3.02	35.88	3.75	p<0.001	DCM > Normal, dFS 18-20%, SAS & dFS<18%. LVE > Normal, dFS18-20% & SAS. dFS<18% > Normal.
LVpws	13.69	1.38	12.74	1.14	12.39	0.92	13.13	1.63	14.72	1.42	13.96	1.50	p<0.001	LVE > dFS<18%, DCM & dFS18-20%. SAS > dFS<18% & DCM. Normal > dFS<18% & DCM.
LVpwd/ LVIDd	0.23	0.03	0.20	0.03	0.23	0.02	0.23	0.03	0.19	0.03	0.22	0.04	p<0.001	dFS18-20% > LVE & DCM. dFS<18% > LVE & DCM. Normal > LVE & DCM
%thIVS	22.97	10.25	18.75	11.79	13.31	7.37	17.68	10.19	27.72	12.91	20.25	11.20	p<0.001	dFS<18% < LVE & Normal
%thLVpw	34.45	10.00	25.42	12.09	28.16	8.11	31.89	9.40	42.15	12.43	34.50	8.31	p<0.001	DCM < LVE, SAS & Normal. dFS<18% < LVE.
FS	24.44	3.21	16.58	3.44	13.89	4.65	18.85	0.51	25.44	2.77	24.50	4.50	p<0.001	LVE > dFS<18%, DCM & dFS18-20%. Normal > dFS<18%, DCM & dFS18-20%. SAS > dFS<18%, DCM & dFS18-20%
EF	46.03	5.10	34.28	6.47	28.88	10.95	39.02	1.32	49.96	4.51	47.73	6.86	p<0.001	LVE > dFS<18%, DCM & dFS18-20%. Normal > dFS<18%, DCM & dFS18-20%. SAS > dFS<18%, DCM & dFS18-20%
R-R	0.59	0.08	0.57	0.09	0.60	0.11	0.62	0.14	0.65	0.11	0.56	0.09	ns	
EPSS	5.68	1.68	8.53	3.02	6.45	2.11	6.14	2.49	7.93	2.12	5.66	2.08	p<0.001	DCM > SAS, Normal & dFS18-20%
EPSS/LVIDd	0.13	0.04	0.17	0.06	0.15	0.05	0.14	0.05	0.15	0.04	0.12	0.04	p<0.001	DCM > Normal & SAS
LAs	24.13	4.06	26.11	3.99	22.41	3.58	22.88	3.22	26.34	5.46	23.77	4.34	p<0.01	No significant pairwise differences identified
Aod	29.18	2.71	31.55	3.43	30.66	3.62	29.33	2.60	28.83	2.40	28.68	2.41	p=0.001	DCM > SAS
LAs:Aod	0.83	0.15	0.84	0.16	0.74	0.14	0.79	0.13	0.93	0.25	0.84	0.19	p<0.05	LVE > dFS<18%
LVId/LVIDd	1.78	0.16	1.60	0.18	1.86	0.18	1.85	0.17	1.45	0.09	1.70	0.15	p<0.001	dFS<18% > LVE, DCM & SAS. dFS18-20% > LVE, DCM & SAS. Normal > LVE, DCM & SAS
MAMseptal	11.29	2.08	9.17	1.80	11.23	2.20	10.78	2.33	10.82	3.29	11.98	2.08	p<0.001	DCM < SAS, Normal & dFS<18%
MAMlateral	13.96	2.27	12.06	2.52	11.62	2.14	12.84	3.23	14.28	1.85	15.17	2.57	p<0.001	SAS > dFS<18%, DCM & dFS18-20%
MAMmean	12.42	1.84	10.15	2.04	11.34	1.82	11.73	2.52	11.77	3.37	13.30	1.95	p<0.001	SAS > DCM, dFS<18%, dFS18-20%. Normal > DCM
M-mode parameters indexed to BSA														
RVd/BSA	5.40	1.87	5.93	2.30	5.16	1.82	5.44	1.39	5.62	1.65	5.53	2.27	ns	
IVSd/BSA	6.97	0.86	7.07	1.30	6.90	1.13	6.97	1.07	7.13	1.00	7.72	1.11	p<0.05	SAS > dFS<18% & Normal
LVIDd/BSA	29.78	3.40	32.74	3.37	27.74	2.36	28.93	2.14	35.77	1.06	32.68	3.59	p<0.001	LVE > dFS<18%, dFS18-20% & Normal DCM > dFS<18%, dFS18-20% & Normal SAS > dFS<18%, dFS18-20% & Normal
LVpwd/BSA	6.72	0.74	6.59	0.91	6.34	0.74	6.76	1.09	6.62	0.61	7.25	0.96	p<0.01	SAS > dFS<18% & DCM
IVSs/BSA	8.44	1.03	8.28	1.23	7.76	1.23	8.01	1.09	9.18	1.16	9.08	1.32	p<0.001	No significant pairwise differences identified
LVIDs/BSA	22.51	2.53	27.24	2.97	23.71	2.13	23.36	1.69	26.70	1.06	24.68	2.25	p<0.001	DCM > Normal, dFS18-20%, dFS<18% & SAS. LVE > Normal, dFS18-20%. SAS > Normal
LVpws/BSA	8.94	0.95	8.24	0.92	8.10	0.93	8.69	1.21	9.53	1.26	9.62	1.29	p<0.001	SAS > dFS<18%, DCM, dFS18-20% and Normal. LVE > dFS<18%. Normal > dFS <18%
LAs/BSA	15.60	2.59	17.22	3.16	14.27	1.81	15.09	2.47	17.12	3.93	16.34	3.07	p<0.01	DCM > dFS<18%
Aod/BSA	19.23	1.77	20.19	1.77	20.15	2.77	19.22	1.63	19.20	2.88	19.67	2.08	ns	

There was no significant difference between the Newfoundland groups for RVd, whether or not indexed to BSA, although the DCM group showed the largest mean value. LVIDd was significantly larger in the DCM, LVE and SAS groups, although the SAS group was not significantly larger than the Normal dogs. Similar results were obtained once LVIDd was indexed to BSA, with the largest value from the LVE group. LVIDs was significantly larger in the DCM group than the Normal, dFS18-20%, SAS and the dFS<18% groups. The LVE group was significantly greater than the Normal, dFS18-20% and the SAS groups and the dFS<18% was significantly greater than the Normal group. Once LVIDs was indexed to BSA, DCM was greater than Normal, dFS18-20%, dFS<18% and SAS groups and the LVE group was significantly larger than Normal and dFS18-20% groups. The SAS group was larger than the Normal group. No significant difference was identified between the groups for IVSd, although once indexed to BSA, the SAS group showed a significantly thicker IVSd/BSA than the Normal group or the dFS<18% group. IVSs was significantly greater for the LVE group (compared with dFS<18%, dFS18-20%, DCM and Normal groups), the SAS group (compared with dFS<18% and dFS18-20% groups) and the Normal group IVSs was significantly thicker than for the dFS<18% group. Once indexed to BSA, although significant differences between groups were identified, no significant pair-wise differences were isolated, although the trend to the greatest value was in the SAS group. LVpwd showed no significant differences between groups, although once indexed to BSA, SAS was greater than dFS<18% and DCM groups. LVpws was significantly greater in the LVE group (compared with the dFS<18%, DCM and dFS18-20% groups), the SAS group and the Normal group (than the dFS<18% and DCM groups). Once indexed to BSA, the LVpws/BSA was significantly greater in the SAS group compared with the dFS<18%, DCM, dFS18-20% and Normal groups and also the LVE and Normal groups compared with the dFS<18% group.

The percentage thickening of the interventricular septum (%thIVS or IVS%th) was significantly lower in the dFS<18% group than the Normal or LVE group. The percentage thickening in the LVpw (%thLVpw or LVpw%th) was significantly lower

in the DCM group compared with the LVE, SAS and Normal groups and the dFS<18% group was also significantly lower than the LVE group.

The use of the parameter obtained by dividing the diastolic LV posterior wall measurement by the LV diastolic diameter measurement (LVpwd/LVIDd) allowed further comparison between groups. The dFS18-20%, dFS<18% and Normal groups had a larger index than the LVE and DCM groups.

The "index of sphericity", determined by LVld:LVIDd, was compared between groups. The dFS<18%, dFS18-20% and the Normal groups all had a greater index than the LVE, the DCM and the SAS group. The mean value for the LVE group was the lowest.

FS% was significantly greater in the LVE, Normal and SAS groups than the DCM group and the two dFS groups. Similar differences were identified for EF when groups were compared. The mean FS and EF were highest in the LVE group, although these findings were not statistically significant.

Mitral M-mode parameters were compared between Newfoundland groups showed that the DCM group had a significantly larger EPSS than the SAS, Normal and dFS<18-20% groups. The LVE group also had a trend to larger EPSS distance although this did not achieve statistical significance. Once the EPSS was indexed to LVIDd, however, the only significant differences were that the DCM EPSS/LVIDd measurement was larger than the Normal or the SAS groups.

When M-mode results obtained at aortic valve level from a short axis view, significant differences were identified between groups (ANOVA $p<0.01$) for LAs, with a trend to higher values in the DCM and the LVE groups. However, multiple pair-wise comparisons failed to identify any two different groups with a p value of less than 0.05. Once normalised to BSA (LAs/BSA), the DCM group had a significantly larger left atrium than the dFS<18% group although there was also a

trend to a large normalised LAs dimension in the LVE group and the SAS group. Aod was identified to be significantly larger in the DCM group than the SAS group, although once normalised to BSA, there were no significant differences between groups. The M-mode ratio of LAs:Aod was significantly larger in the LVE group than the dFS<18% group.

Significant differences between Newfoundland groups were identified for mitral annulus motion. MAMseptal was significantly less in the DCM group than the SAS, Normal and dFS<18% groups. MAMlateral was significantly greater in the SAS group than the dFS<18%, DCM and dFS18-20% groups. In all groups, MAMlateral was larger than MAMscptal, although MAMseptal could be more consistently obtained. Mean mitral annulus motion (MAMmean) was significantly less in the DCM group than SAS or Normal Newfoundlands. The SAS group also had significantly greater MAMmean than dFS<18% and dFS18-20% groups.

B.24.6. *Sub-aortic stenosis group*

When linear regression was used to screen for any relationship between the various 2D and M-mode echocardiographic parameters or the R-R intervals and the sub-costal peak aortic velocity, none was found.

B.24.7.1. *Aortic flow parameters*

Descriptive statistics for the various parameters of aortic flow for all Newfoundland groups are displayed in Table B.16. Results are also shown for ANOVA or ANOVA on ranks, with multiple pair-wise comparison procedures performed by the Tukey test or Dunn's method respectively. Box and whisker plots are illustrated in the Supplement (available from the author).

Table B.16.
Means and Standard deviations for Newfoundland groups with One Way Analysis of Variance to identify differences between groups: echo-Doppler parameters (Aortic flow)

	NORMAL Group		DCM group		dFS<18% group		dFS18-20 group		LVE group		SAS group		ANOVA or ANOVA on RANKS	Multiple Pairwise comparisons: (TUKEY test or DUNNS method)
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd		
Aortic Doppler parameters														
Subcostal view														
Aov	1.48	0.15	1.30	0.23	1.30	0.20	1.37	0.16	1.39	0.20	1.94	0.37	p<0.001	SAS > dFS<18%, DCM, dFs18-20%, LVE & Normal
Aovti	0.17	0.02	0.14	0.03	0.15	0.03	0.16	0.02	0.17	0.03	0.22	0.04	p<0.001	SAS > DCM, dFS<18%, dFS18-20%, LVE & Normal Normal > dFS<18% & DCM.
Aodv/dtmax	46.89	8.84	36.66	8.02	37.71	8.07	38.83	6.05	45.92	10.18	57.41	12.88	p<0.001	SAS > DCM, dFS<18%, dFS18-20% Normal > DCM, dFS<18% & dFS18-20%.
Aodv/dtmean	27.69	4.50	21.37	4.65	22.34	4.51	22.59	3.57	27.50	7.09	26.64	7.94	p<0.001	SAS > DCM, dFS<18%, dFS18-20%, LVE & Normal. Normal > DCM, dFS<18% & dFS18-20%.
cPEP	0.072	0.010	0.094	0.012	0.084	0.011	0.081	0.012	0.082	0.006	0.065	0.010	p<0.001	LVE & DCM > SAS, Normal, dFS18-20% & dFS<18%. dFS<18% & dFS18-20% > SAS & Normal. Normal > SAS.
ET	0.178	0.013	0.166	0.015	0.169	0.014	0.180	0.019	0.186	0.008	0.171	0.016	p<0.001	DCM < LVE, dFS18-20% & Normal.
Acc.t	0.054	0.006	0.059	0.007	0.057	0.006	0.058	0.006	0.057	0.006	0.053	0.008	p<0.001	DCM > SAS & Normal
cPEP:ET ratio	0.403	0.057	0.569	0.100	0.489	0.086	0.449	0.070	0.440	0.042	0.379	0.064	p<0.001	DCM & dFS<18% > SAS & Normal. dFS18-20% > SAS.
Vcf	1.38	0.20	1.00	0.20	0.79	0.27	1.05	0.10	1.36	0.14	1.43	0.23	p<0.001	SAS, Normal & LVE > dFS<18%, DCM & dFS18-20%. dFS18-20% > dFS<18%. DCM > dFS< 18%.
HR	97.06	15.41	100.61	14.99	105.20	31.76	96.32	18.19	87.71	11.32	109.85	18.88	p<0.01	SAS > LVE
R-R	0.64	0.11	0.61	0.09	0.60	0.12	0.65	0.13	0.69	0.09	0.56	0.11		
Left apical view														
L.Aov	1.17	0.14	1.03	0.18	1.04	0.16	1.12	0.19	1.11	0.20	1.51	0.35	p<0.001	SAS > DCM, dFS<18%, dFS18-20%, LVE & Normal. Normal > DCM & dFS<18%.
Intervals indexed to square root of R-R														
cPEP/$\sqrt{R-R}$	0.091	0.012	0.122	0.019	0.112	0.022	0.102	0.012	0.100	0.006	0.086	0.011	p<0.001	DCM & dFS<18% > SAS & Normal. dFS18-20% > SAS
ET/$\sqrt{R-R}$	0.225	0.014	0.214	0.02	0.222	0.026	0.225	0.018	0.224	0.014	0.230	0.019	p<0.05	DCM < SAS & Normal.
acc.t/$\sqrt{R-R}$	0.068	0.008	0.077	0.011	0.074	0.010	0.073	0.009	0.069	0.008	0.071	0.011	p=0.001	DCM > Normal
cPEP:ET/$\sqrt{R-R}$	0.515	0.085	0.742	0.158	0.666	0.171	0.576	0.106	0.538	0.053	0.508	0.090	p<0.001	DCM > SAS, Normal, LVE, dFS18-20%. dFS<18% > SAS & Normal
Vcf/$\sqrt{R-R}$	1.76	0.35	1.27	0.30	1.04	0.43	1.35	0.24	1.66	0.17	1.93	0.37	p<0.001	SAS & Normal > dFS<18%, DCM & dFS18-20%. LVE > dFS<18%

Aov and Aovti were significantly greater (by definition) in the SAS group than all other Newfoundland groups. No other significant differences were identified for Aov. Aovti was also significantly greater in Normal Newfoundlands than the dFS<18% and DCM groups. Peak acceleration of aortic flow (dv/dt_{max}) was significant greater in the SAS and Normal groups than DCM, dFS<18% and dFS18-20% groups. The mean acceleration of aortic flow (dv/dt_{mean}) was significantly greater in the SAS group than all other Newfoundland groups and Normal Newfoundlands had greater dv/dt_{mean} than dogs in DCM, dFS<18% and dFS18-20% groups.

There was a significant difference in heart rate determined between the groups during recording of subcostal aortic flow spectra ($p<0.01$), with the SAS group having a significantly higher heart rate than the LVE group. STIs were corrected for heart rate by indexing by √R-R interval.

PEP was significantly greater in the DCM and LVE groups than the other Newfoundland groups. The PEP of the dFS<18% and dFS18-20% was significantly longer than the SAS and Normal groups, and SAS PEP was significantly shorter than the Normal group. PEP/√R-R was significantly greater in the DCM and dFS<18% groups than the SAS and Normal groups, and the dFS18-20% group had a significantly greater value than the SAS group.

ET was significantly shorter in the DCM group than the LVE, dFS18-20% and Normal groups (Figure B.12.g.). After correction for heart rate, ET/√R-R was significantly less in the DCM group than the SAS and Normal Newfoundlands. Aortic flow acceleration time was significantly longer in the DCM group than the SAS and normal Newfoundlands. These trends were still apparent after normalising to √R-R.

The PEP:ET ratio was significantly longer in the DCM and dFS<18% groups than the SAS and Normal dogs. PEP:ET/√R-R was significantly greater in the DCM Newfoundlands than the SAS, Normal, LVE and dFS18-20% groups. Vcf was

significantly greater in the SAS, Normal and LVE groups than the dFS<18%, DCM and dFS18-20% groups. After indexing to √R-R, the SAS and Normal dogs were shown to be significantly different from dFS<18%, DCM and dFS18-20% groups, although the LVE group was also significantly greater than the dFS<18% group.

B.24.7.2. *Sensitivity and Specificity and Predictive value of PEP or the PEP:ET ratio in distinguishing between Normal and DCM Newfoundlands.*

The normal PEP(corrected) (mean ± sd) was 0.072 ± 0.010 seconds. Two cut-off values were assessed in order to differentiate between Normal and DCM Newfoundlands:

(i) Mean + 2sd = values > 0.092 (positive (+ve) for DCM) and ≤ 0.092 (negative (-ve) for DCM).

(ii) Mean + 1sd = values > 0.082 (+ve for DCM) and ≤ 0.082 (-ve for DCM).

The normal (corrected) PEP:ET ratio (mean ± sd) was 0.403 ± 0.057. Two cut-offs were assessed in an attempt to differentiate between Normal and DCM Newfoundlands:

(iii) Mean + 2sd = values > 0.517 (+ve for DCM) and ≤ (-ve for DCM).

(iv) Mean +1sd = values > 0.460 (+ve for DCM) and ≤ (-ve for DCM).

Results for these four different criteria are displayed in Table B.17.

Table B.17.

Data for calculating sensitivity and specificity for differentiating between Normal and DCM Newfoundlands based on systolic time interval criteria

(i) PEP>0.092 s		**DCM**	**Normal**	Total
	+ve	21	1	22
	-ve	13	81	94
	Total	34	82	**116**
(ii) PEP>0.082 s		**DCM**	**Normal**	Total
	+ve	27	11	38
	-ve	7	71	78
	Total	34	82	**116**
(iii) PEP:ET > 0.517		**DCM**	**Normal**	Total
	+ve	21	1	22
	-ve	13	81	94
	Total	34	82	**116**
(iv) PEP:ET > 0.460		**DCM**	**Normal**	Total
	+ve	29	14	43
	-ve	5	68	73
	Total	34	82	**116**

(i) **PEP>0.92s.**

Sensitivity = 21/34 = 61.7%
Specificity = 81/82 = 98.8%
Positive Predictive value = 21/22 = 95.5%
Negative Predictive value = 81/94 = 86.2%

(ii) **PEP>0.082s**

Sensitivity = 27/34 = 79.4%
Specificity = 71/82 = 86.5%
Positive Predictive value = 27/38 = 71.1%
Negative Predictive value = 71/78 = 91.0%

(iii) **PEP:ET > 0.517**

Sensitivity = 21/34 = 61.7%
Specificity = 81/82 = 98.8%
Positive Predictive value = 21/22 = 95.5%
Negative Predictive value = 81/94 = 86.2%

(iv) **PEP:ET > 0.460**

Sensitivity = 29/34 = 85.3%
Specificity = 68/82 = 82.9%
Positive Predictive value = 29/43 = 67.4%
Negative Predictive value = 68/73 = 93.2%

If the criterion of PEP:ET ratio greater than one standard deviation above the Normal mean (>0.460) was applied to the other Newfoundland groups, the following "positive" results were identified. There were 14/82 positives in the Normal Newfoundlands (17.1%), 29/34 positives in the DCM group (85.3%), 15/29 positives in the dFS<18% group (51.7%), 11/24 positives in the dFS18-20% (45.8%), 2/8 positives in the LVE group (25%) and 1/40 positives in the SAS group (2.5%).

B.24.8. *Aortic insufficiency*

Aortic insufficiency was identified in a number of Newfoundlands by CFDE, scored as previously noted (B.15.1). Results are presented in Table B.18.

Table B.18.
Newfoundlands in different categories with aortic insufficiency grades

Newfoundland Group	0	0.5	1	2	TOTAL
Normal	64 (74.4%)	7 (8.1%)	14 (16.3%)	1 (1.2%)	86
DCM	29 (82.9%)	2 (5.7%)	4 (11.4%)	0 (0%)	35
dFS<18%	26 (89.7%)	0 (0%)	2 (6.9%)	1 (3.4%)	29
dFS18-20%	17 (70.8%)	3 (12.5%)	4 (16.7%)	0 (0%)	24
LVE	6 (75.0%)	0 (0%)	2 (25.0%)	0 (0%)	8
SAS	13 (32.5%)	3 (7.5%)	21 (52.5%)	3 (7.5%)	40

χ^2 analysis showed that there was significantly more aortic regurgitation in the SAS group than Normal Newfoundlands ($p<0.001$). There were no significant differences identified between Normal Newfoundlands and any of the other groups.

Linear regression was used to attempt to identify any significant relationship between aortic velocity (CW, subcostal) and the grade of aortic insufficiency. No association was identified in the Normal, DCM, dFS18-20% or the LVE groups. However, a weak but significant ($p<0.05$) relationship was identified for the dFS<18% group, given by the equation:

$$\text{dFS<18\% S/C Aov} = 1.268 + (0.169 \times \text{AoI grade})$$

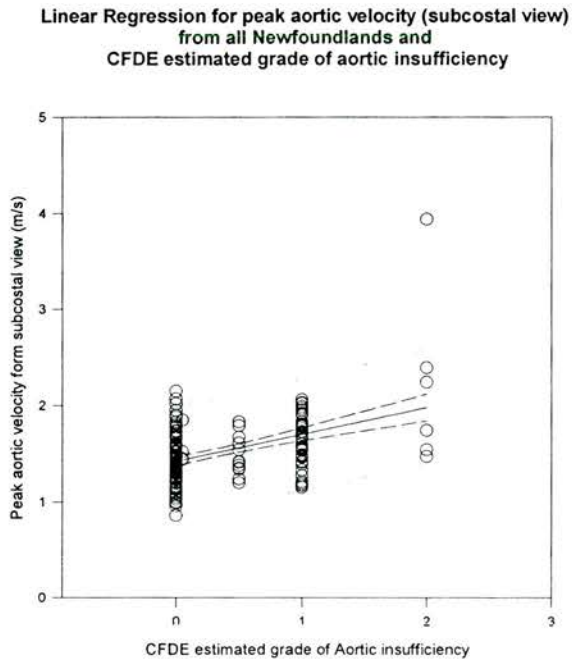
($R=0.433$, $R^2 = 0.187$, adjusted $R^2 = 0.147$, $p=0.044$).

A more significant relationship was identified between aortic velocity and the severity of aortic regurgitation for the SAS group ($p<0.01$). This is given by the equation:

$$\text{SAS S/C Aov} = 1.767 + (0.255 \times \text{AoI grade})$$

($R=0.422$, $R^2 = 0.178$, adjusted $R^2 = 0.157$, $p=0.007$)

When all data was grouped from all Newfoundland groups (n=193), the relationship of S/C CW aortic velocity to grade of aortic insufficiency was investigated in a similar manner by linear regression, and was found to be highly significant ($p<0.001$). The graph and equation are shown in Figure B.3.



Linear regression for subcostal CW peak aortic velocity and the grade of aortic insufficiency determined by colour flow Doppler echocardiography (CFDE). The equation is:

S/C Aov = 1.419 + (0.278 x AoI grade)

($R=0.445$, $R^2=0.198$, adjusted $R^2=0.194$ and $p<0.001$).

Figure B.3.
Linear regression for peak aortic velocity (subcostal view) from all Newfoundlands and CFDE estimated grade of aortic insufficiency

B.24.9. *Mitral inflow parameters*

Descriptive statistics for the various parameters of mitral flow for all Newfoundland groups are displayed in Table B.19. Results are also shown for ANOVA or ANOVA on ranks, with multiple pair-wise comparison procedures performed by the Tukey test or Dunn's method respectively. Box and whisker plots are available in the Supplement (available from the author). There was a significant difference in heart rate identified between the groups during the mitral flow recordings, with SAS Newfoundlands having a significantly higher heart rate than the LVE group.

Figure B.19.
Means and Standard deviations for Newfoundland groups
with One Way Analysis of Variance to identify differences between groups:
echo-Doppler parameters (Mitral valve)

	NORMAL Group		DCM group		dFS<18% group		dFS18-20 group		LVE group		SAS group		ANOVA or ANOVA on RANKS	Multiple Pairwise comparisons: (TUKEY test or DUNNS method)
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd		
Mitral Doppler parameters														
Mitral Ev	0.61	0.13	0.66	0.16	0.55	0.13	0.56	0.12	0.62	0.09	0.69	0.14	p<0.001	SAS > dFS<18%., dFS18-20% & Normal. DCM > dFS<18% & dFS18-20%.
Mitral Av	0.48	0.10	0.42	0.09	0.40	0.09	0.44	0.10	0.47	0.13	0.54	0.13	p<0.001	SAS > dFS<18%, DCM & dFS18-20%. Normal > dFS<18%
Mitral E:A	1.32	0.40	1.46	0.39	1.41	0.42	1.35	0.49	1.48	0.68	1.33	0.35	ns	
Mitral Evti	0.063	0.011	0.064	0.012	0.058	0.013	0.060	0.014	0.061	0.014	0.067	0.013	ns	
Mitral Avti	0.022	0.007	0.017	0.008	0.019	0.006	0.022	0.007	0.021	0.011	0.024	0.008	p<0.01	No significant pairwise differences.
Mitral Evti:Avti	3.09	1.17	4.30	2.20	3.51	1.57	2.99	1.34	2.61	0.98	3.08	1.29	ns	
total vti (E+A)	0.086	0.014	0.079	0.012	0.076	0.015	0.082	0.016	0.083	0.016	0.090	0.018	p<0.05	SAS > dFS<18%
Ev/total vti	7.15	1.28	7.75	1.59	7.31	1.34	6.91	1.11	7.65	1.59	7.78	1.47	ns	
Av/total vti	5.63	1.08	5.48	1.23	5.39	1.10	5.58	1.55	5.62	1.36	6.10	1.67	ns	
Evti/total vti	0.74	0.07	0.78	0.08	0.76	0.07	0.73	0.08	0.75	0.13	0.74	0.06	ns	
Avti/total vti	0.26	0.07	0.22	0.08	0.24	0.07	0.27	0.08	0.25	0.13	0.26	0.06	ns	
E deceleration t	0.117	0.018	0.113	0.024	0.121	0.021	0.114	0.017	0.124	0.016	0.106	0.018	p<0.05	No significant pairwise differences.
E decel/√R-R	0.150	0.022	0.151	0.031	0.154	0.024	0.145	0.023	0.152	0.019	0.144	0.027	ns	
E duration	0.192	0.022	0.186	0.032	0.192	0.026	0.188	0.023	0.193	0.018	0.174	0.027	p<0.01	No significant pairwise differences.
E dur /√R-R	0.249	0.031	0.248	0.040	0.248	0.030	0.240	0.025	0.235	0.021	0.236	0.037	ns	
A duration	0.090	0.012	0.087	0.014	0.088	0.013	0.092	0.013	0.084	0.025	0.086	0.015	ns	
A dur /√R-R	0.116	0.015	0.112	0.017	0.113	0.015	0.118	0.015	0.103	0.031	0.115	0.016	ns	
HR	100.62	19.72	108.83	21.79	100.79	14.31	100.38	17.94	89.86	6.72	112.45	20.27	p<0.01	SAS > LVE
R-R	0.61	0.10	0.57	0.11	0.61	0.09	0.62	0.11	0.67	0.05	0.55	0.10		
Isovolumic relaxation time														
IVRT	0.062	0.010	0.070	0.027	0.064	0.008	0.066	0.014	0.069	0.018	0.060	0.012	ns	
IVRT /√R-R	0.080	0.013	0.095	0.039	0.081	0.012	0.084	0.017	0.085	0.021	0.080	0.018	ns	
HR	101.24	18.07	111.27	21.52	97.35	15.18	99.79	20.13	91.63	11.06	107.34	17.81	p<0.05	No significant pairwise differences.
R-R	0.61	0.11	0.56	0.10	0.63	0.11	0.62	0.12	0.66	0.08	0.57	0.10		

Mitral Ev was significantly higher in the SAS group than the dFS<18%, dFS18-20% groups and in Normal Newfoundlands. It was higher in the DCM group than these groups also, although this was only statistically significant for the depressed fractional shortening groups. Av was significantly higher in the SAS group than the DCM group and both depressed fractional shortening groups and was significantly higher in Normal Newfoundlands than Newfoundlands with dFS<18%. There was no significant difference between groups for the E:A velocity ratio, although it was higher in the DCM and LVE groups than the other groups.

Evti was not significantly different between Newfoundland groups. Although a significant ($p<0.01$) ANOVA result was recorded for Avti, no significant pair-wise differences were identified in the multiple comparison procedure. There was no significant differences between groups for the Evti:Avti ratio. The total mitral vti was simply calculated by adding Evti and Avti. The SAS group showed a significantly higher total vti than the dFS<18% group, although the DCM group also had a similarly low value to the dFS<18% group. When Ev or Av was normalised to the total vti, no significant differences were evident between the groups for either parameter. If the proportions of early left ventricular filling (Evti / total vti) or late ventricular filling (Avti / total vti) were compared between groups, no significant differences were identified.

Mitral E duration was significantly different between the groups, although no significant pair-wise differences were identified. However, after normalising this time to heart rate by dividing by √R-R interval, differences between the groups were no longer evident. E deceleration time was significantly different between groups, although no significant pair-wise differences were identified. However, after indexing E deceleration/√R-R interval, no significant differences were identified between the Newfoundland groups. Mitral A duration, whether or not indexed to √R-R, was not significantly different between Newfoundland groups.

No significant differences were identified between Newfoundland groups for IVRT or IVRT/√R-R.

The results for mitral regurgitation were recorded previously in Section B.24.4.1 and B.24.4.2.

B.24.10. *Pulmonary venous flow*

Descriptive statistics for the various parameters of pulmonary venous flow for all Newfoundland groups are displayed in Table B.20. Results are also shown for ANOVA or ANOVA on ranks, with multiple pair-wise comparison procedures performed by the Tukey test or Dunn's method respectively. Box and whisker plots available in the Supplement (available from the author). During the recordings of PVF, a statistically significant difference between heart rates of the Newfoundland groups was identified ($p<0.01$), which was due to the DCM group having a significantly faster rate than the LVE group.

Table B.20.
Means and Standard deviations for Newfoundland groups with One Way Analysis of Variance to identify differences between groups: echo-Doppler parameters (Pulmonary venous flow)

	NORMAL Group		DCM group		dFS<18% group		dFS18-20 group		LVE group		SAS group		ANOVA or ANOVA on RANKS	Multiple Pairwise comparisons: (TUKEY test or DUNNS method)
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd		
PVF Doppler parameters														
PVFArv	0.24	0.11	0.21	0.06	0.19	0.05	0.22	0.07	0.20	0.02	0.26	0.09	p<0.01	SAS > dFS<18%
PVF Sv	0.38	0.06	0.41	0.13	0.38	0.08	0.36	0.06	0.36	0.08	0.41	0.11	ns	
PVF Dv	0.37	0.05	0.43	0.12	0.38	0.06	0.37	0.06	0.40	0.13	0.41	0.07	p<0.05	No significant pairwise differences.
PVF R2v	0.16	0.05	0.18	0.08	0.15	0.03	0.14	0.03	0.16	0.02	0.18	0.05	p<0.05	SAS > dFS18-20%
PVF Svti	0.063	0.012	0.054	0.016	0.058	0.013	0.057	0.015	0.064	0.013	0.066	0.017	p<0.05	No significant pairwise differences.
PVF Dvti	0.082	0.020	0.084	0.026	0.086	0.020	0.091	0.033	0.092	0.039	0.093	0.031	ns	
PVF Ar dur	0.067	0.013	0.066	0.014	0.069	0.011	0.072	0.011	0.068	0.008	0.069	0.010	ns	
PVF Ar dur/√R-R	0.084	0.017	0.086	0.020	0.085	0.014	0.090	0.016	0.081	0.012	0.089	0.014	ns	
PVF S dur	0.248	0.032	0.229	0.041	0.244	0.025	0.251	0.025	0.286	0.030	0.246	0.031	p=0.001	LVE > DCM, dFS<18%, SAS & Normal
PVF S dur/√R-R	0.310	0.039	0.303	0.038	0.301	0.032	0.311	0.035	0.336	0.038	0.316	0.042	ns	
PVF D dur	0.339	0.060	0.314	0.061	0.349	0.059	0.346	0.074	0.359	0.054	0.328	0.052	ns	
PVF D dur/√R-R	0.424	0.052	0.407	0.067	0.429	0.060	0.423	0.067	0.419	0.037	0.417	0.047	ns	
PVF D decel.t	0.199	0.049	0.188	0.051	0.209	0.051	0.194	0.043	0.236	0.063	0.185	0.048	ns	
PVF D decel/√R-R	0.248	0.052	0.245	0.058	0.257	0.054	0.238	0.045	0.274	0.050	0.237	0.050	ns	
PVF R2 dur	0.062	0.009	0.057	0.009	0.059	0.006	0.057	0.008	0.055	0.007	0.067	0.017	ns	
PVF R2 dur/√R-R	0.078	0.012	0.075	0.014	0.074	0.010	0.072	0.014	0.067	0.012	0.090	0.024	ns	
HR	95.75	14.29	104.24	16.29	92.55	13.91	93.55	18.29	83.86	13.93	99.81	15.07	p<0.01	DCM > LVE
R-R	0.64	0.10	0.59	0.09	0.66	0.10	0.66	0.12	0.73	0.14	0.62	0.10		
Sv/Dv	1.04	0.19	0.98	0.28	1.02	0.21	0.98	0.21	0.95	0.22	1.00	0.26	ns	
Svti/Dvti	0.81	0.26	0.70	0.27	0.73	0.29	0.73	0.42	0.76	0.21	0.76	0.28	ns	
Svti/total vti	0.437	0.077	0.396	0.095	0.408	0.091	0.397	0.111	0.423	0.074	0.419	0.083	ns	
total forward vti	0.145	0.023	0.138	0.031	0.144	0.019	0.148	0.032	0.156	0.045	0.158	0.037	ns	

Arv was significantly higher in the SAS group than the dFS<18% group. There were no significant differences between groups apparent when Sv was considered. Significant differences between Newfoundland groups were apparent for Dv velocity ($p<0.05$), although no significant pair-wise differences were identified. The DCM group appeared to have a wider range of D wave velocities, with a trend to higher velocity than the other groups. There were no statistically significant differences between groups for the S:D velocity ratio. R2v was significantly greater in the SAS group than the dFS18-20% group, although a wide range of velocities with a tendency to higher R2 wave velocities was visually evident in the DCM group.

Svti was significantly different for the Newfoundland groups, although no pair-wise differences were identified. However, a trend to lower Svti in the DCM group was identified, and the higher Svtis occurred in the LVE and SAS groups. There was no significant differences between Newfoundland groups for Dvti or the S:D vti ratio.

The total forward pulmonary venous flow was determined by summing S vti and D vti. Although there were no statistically significant differences evident between groups, the DCM group had the lowest PVF total vti and Normal dogs showed the highest mean value. The systolic fraction of total forward pulmonary venous flow was calculated as Svti/total vti. Although there were no statistically significant differences identified in the Newfoundland groups with respect to this parameter, a trend to a lower value was identified in the DCM and dFS<18% groups and the maximal mean value was in Normal Newfoundlands.

PVF wave durations were compared for Newfoundland groups, uncorrected and corrected for heart rate (by dividing by the $\sqrt{}$R-R interval). S duration was significantly longer in the LVE group than the DCM, dFS<18%, SAS and Normal dogs. However, this difference was largely determined by the heart rate differences between the groups, as no statistically significant difference between the groups was identified after indexing to $\sqrt{}$R-R, although the LVE group still tended to show the highest values. No significant differences between Newfoundland groups were

identified for Ar, D or R2 durations, or the D deceleration time, whether or not these parameters were indexed to √R-R.

There was no significant difference between Newfoundland groups for the difference between mitral A duration and PVF Ar duration (data not shown). In almost all cases, mitral A wave duration exceeded PVF Ar duration. There were two exceptions in each of the five abnormal groups, with differences ranging from -0.003 to -0.02 seconds. As most differences were less than ten milliseconds (the temporal resolution of a Doppler system), these differences are not significant.

Although a significant relationship had been demonstrated between the left atrial emptying index (LAEI) and S:D vti ratio in Normal Newfoundlands, no significant association between these two parameters was identified for any of the other Newfoundland groups. The LAEI showed no significant relationship to any of the PVF parameters for any of the other Newfoundland groups.

B.24.11. *Pulmonary Artery Parameters*

Descriptive statistics for pulmonary artery flow velocity for all Newfoundland groups are displayed in Table B.21. Results are also shown for ANOVA or ANOVA on ranks, with multiple pair-wise comparison procedures performed by the Tukey test or Dunn's method respectively. Box and Whisker plots are available in the Supplement (available from the author). The heart rate was significantly different between Newfoundland groups during recording of pulmonary artery signals. From the RPS view, the heart rate of the SAS group was significantly greater than the LVE group, and from the LPS view, the SAS group had significantly faster heart rates than the LVE and the dFS<18% groups.

The SAS group had significantly higher PAv than all other groups, including normal Normal Newfoundlands. Normal Newfoundlands had higher PAv than the DCM or dFS<18% groups, from both RPS and LPS windows.

Table B.21.
Means and Standard deviations for Newfoundland groups with One Way Analysis of Variance to identify differences between groups: echo-Doppler parameters (Pulmonary artery and tricuspid inflow)

	NORMAL Group		DCM group		dFS<18% group		dFS18-20 group		LVE group		SAS group		ANOVA or ANOVA on RANKS	Multiple Pairwise comparisons: (TUKEY test or DUNNS methoc)
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd		
RPS Pulm. flow parameters														
PAv	0.72	0.12	0.62	0.17	0.60	0.09	0.67	0.11	0.65	0.11	0.86	0.14	p<0.001	SAS > dFS<18%, DCM, LVE, dFS18-20% & Normal. Normal > dFS<18% & DCM
HR	96.94	7.01	100.37	16.59	96.04	15.42	96.62	14.21	78.88	12.08	104.94	17.94	p<0.01	SAS > LVE
R-R	0.64	0.11	0.61	0.10	0.64	0.10	0.64	0.10	0.78	0.13	0.59	0.10		
LPS Pulm. flow parameters														
PAv	0.75	0.13	0.64	0.16	0.64	0.09	0.73	0.15	0.66	0.08	0.88	0.13	p<0.001	SAS > DCM, dFS<18%, LVE, dFS18-20% & Normal. Normal > DCM & dFS<18%
HR	96.73	15.30	103.03	14.98	93.61	13.11	95.35	19.10	85.86	10.38	105.44	17.75	p<0.01	SAS > LVE & dFS<18%
R-R	0.64	0.11	0.60	0.08	0.65	0.09	0.65	0.13	0.71	0.08	0.59	0.09		
Tricuspid inflow parameters														
TEv	0.49	0.08	0.44	0.10	0.45	0.07	0.50	0.10	0.43	0.08	0.56	0.11	p<0.001	SAS > LVE, DCM, dFS<18% & Normal
TAv	0.34	0.08	0.29	0.07	0.33	0.07	0.33	0.10	0.30	0.08	0.41	0.11	p<0.001	DCM < SAS & Normal.
TEv:TAv	1.48	0.37	1.48	0.41	1.39	0.31	1.62	0.49	1.47	0.28	1.43	0.39	ns	
HR	97.11	15.63	100.36	14.74	95.36	14.00	93.48	18.64	87.20	4.87	105.15	15.50	p<0.05	No significant pairwise differences.
R-R	0.64	0.1	0.61	0.09	0.64	0.09	0.67	0.13	0.69	0.04	0.58	0.08		

B.24.11.1. *Relationship between pulmonary artery and aortic flow velocities*

Linear regression was used to attempt to identify a relationship between LPS PAv and subcostal Aov from the SAS group. A weak but significant relationship ($p<0.05$) was identified and given by the equation:

$$\text{LPS PAv} = 0.613 + (0.136 \times \text{S/C Aov})$$

($R = 0.386$, $R^2 = 0.149$, adjusted $R^2 = 0.126$ and $p = 0.015$).

The general relationship between pulmonary artery velocity and aortic velocity was investigated by combining the data from all Newfoundland groups, and linear regression was used to identify any association between RPS or LPS PAv and CW Aov obtained from the subcostal view. The linear regression graphs are shown in Figures B.4.a and B.4.b. A moderate correlation which was highly significant was identified for both relationships ($p<0.001$ in both cases).

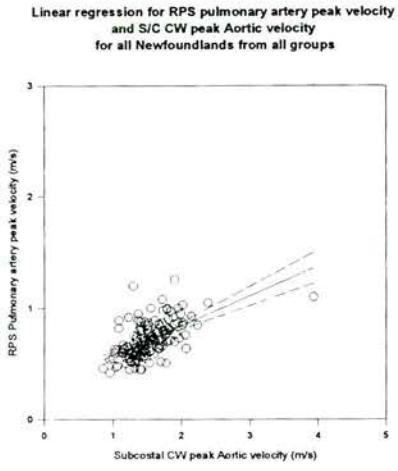


Figure B.4.a.

Linear regression for RPS pulmonary artery peak velocity and subcostal CW aortic velocity for all Newfoundlands from all groups.

Equation:

RPS PAv = 0.309 + (0.267 x S/C Aov)
($R=0.576$, $R^2 = 0.332$, adjusted $R^2 = 0.328$, $p<0.001$)

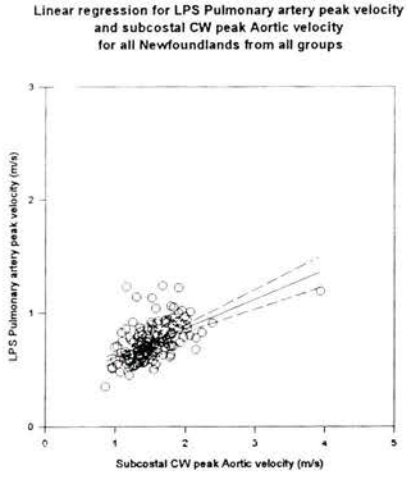


Figure B.4.b.

Linear regression for LPS pulmonary artery peak velocity and subcostal CW aortic velocity for all Newfoundlands from all groups.

Equation:

LPS PAv = 0.356 + (0.255 x S/C Aov)
($R=0.546$, $R^2 = 0.298$, adjusted $R^2 = 0.294$, $p<0.001$)

Figures B.4.

Relationship between Pulmonary artery velocity and Aortic velocity for all Newfoundlands using combined data from all groups.

B.24.11.2. *Pulmonic insufficiency*

Pulmonic insufficiency was identified by CFDE in many Newfoundlands in all groups. This was scored as previously described. Results are given in Table B.22.

Table B.22.
Newfoundlands in different categories with pulmonic insufficiency grades

Newfoundland Group	0	0.5	1	2	TOTAL
Normal	60 (69.8%)	16 (18.7%)	8 (9.3%)	2 (2.3%)	86
DCM	25 (71.4%)	4 (11.4%)	6 (17.1%)	0 (0%)	35
dFS<18%	21 (72.4%)	1 (3.3%)	7 (23.3%)	0 (0%)	29
dFS18-20%	12 (50%)	1 (4.2%)	7 (29.2%)	4 (16.7%)	24
LVE	5 (62.5%)	0 (0%)	3 (37.5%)	0 (0%)	8
SAS	23 (57.5%)	3 (7.5%)	12 (30%)	2 (5%)	40

χ^2 analysis showed that there was significantly more pulmonic insufficiency in the dFS18-20% group ($p=0.001$) and the SAS group ($p=0.013$) than the normal Newfoundlands. No significant differences were identified between the DCM, LVE and dFS<18% groups and Normal Newfoundlands.

B.24.12. *Tricuspid valve parameters*

Descriptive statistics for the parameters of tricuspid inflow for all Newfoundland groups are displayed in Table B.21. Results are also shown for ANOVA or ANOVA on ranks, with multiple pair-wise comparison procedures performed by the Tukey test or Dunn's method respectively. There was a significant difference in heart rate between the groups, with a trend to faster rate in the SAS group and the lowest heart rate in the LVE group.

Tricuspid Ev was significantly higher in the SAS group than most other Newfoundland groups. Tricuspid Av was less in the DCM group than the Normal or SAS groups. There was no statistically significant relationship identified between Newfoundland groups with respect to the tricuspid E:A velocity ratio.

B.24.12.1 *Tricuspid regurgitation*

A number of Newfoundlands had tricuspid regurgitation identified by CFDE, which was scored as previously described (Table B.23.).

Table B.23.

Newfoundlands in different categories with tricuspid regurgitation grades

Newfoundland Group	0	0.5	1	2	TOTAL
Normal	65 (75.6%)	11 (12.8%)	9 (10.5%)	1 (1.2%)	86
DCM	23 (63.9%)	2 (5.6%)	9 (25%)	2 (5.6%)	35
dFS<18%	22 (75.8%)	2 (6.7%)	4 (13.3%)	1 (3.3%)	29
dFS18-20%	20 (83.3%)	1 (4.2%)	3 (12.5%)	0 (0%)	24
LVE	8 (100%)	0 (0%)	0 (0%)	0 (0%)	8
SAS	32 (80%)	4 (10%)	3 (7.5%)	1 (2.5%)	40

χ^2 analysis showed that there was significantly more tricuspid regurgitation in the DCM group than Normal Newfoundlands (p=0.039). No significant differences were identified between any of the other groups and Normal Newfoundlands.

B.25. *Effect of independent variables HR, BSA, Wt, Age or Gender on Echocardiographic parameters for different Newfoundland groups*

B.25.1. *DCM group*

B.25.1.1. *2D Parameters*

The 2D echocardiographic parameters for left ventricle and left atrium are detailed in Table B.24. (Volume II) and graphs are illustrated in the Supplement (available from the author). LVdv and LVsv were significantly associated with BSA or body weight. Normalisation to BSA showed that the end-systolic volume index (ESVI) was significantly larger in male Newfoundlands with DCM than females. Most left atrial measurements were not related to any of the independent variables, although weak but significant ($p<0.05$) relationship was shown between LAls and Weight or BSA. The 2D Aod increased significantly with body weight or BSA.

B.25.1.2. *M-mode parameters*

The relationship between some of the independent variables and various M-mode echocardiographic parameters for the DCM group are shown Table B.25. (Volume II). Graphs are illustrated in the Supplement (available from the author). Both LVIDd and LVIDs showed weak but significant relationships with BSA or weight. No relationships were identified for IVSd or IVSs, although IVSs normalised to BSA showed a significant trend to thinner walls in male Newfoundlands with DCM. No relationships were identified for the LVpwd or LVpws although the LVpwd indexed to BSA showed a significant trend to increasing wall thickness with advancing age. M-mode LAs, once indexed to BSA, showed a significant increasing size with advancing age. M-mode Aod was positively associated with BSA or weight. The M-mode ratio of LAs:Aod was shown to be influenced most significantly by gender, with a larger ratio in male Newfoundlands with DCM. Other variables were eliminated in the forward stepwise or multiple linear regression models, although simple linear regression equations showed weak but significant negative association between LAs:Aod and BSA or weight and a significant positive association between

LAs:Aod and advancing age. MAMseptal was not significantly influenced by any parameter, although age had been initially identified in the forward stepwise regression. MAMlateral was positively influenced by body weight or BSA No significant variable influenced MAMmean in the DCM group.

B.25.1.3. *Doppler parameters of aortic flow*

The Doppler parameters of aortic flow were not greatly affected by independent variables in the DCM group. The significant equations are shown in Table B.26. (Volume II) and linear regression graphs are shown in the Supplement (available from the author). The aortic vti tended to decrease with advancing age. There was an age related increase in the PEP:ET ratio before and after indexing to √R-R and Vcf and Vcf/√R-R tended to increase with age.

B.25.1.4. *Doppler parameters of mitral flow*

The mitral inflow spectral Doppler parameters were also assessed. Results are shown in Table B.27. (Volume II). Graphs are available in the Supplement (available from the author). The mitral E:A velocity ratio was positively related to body weight. Ev indexed to total mitral velocity time integral (Ev/total vti) also tended to increase in heavier dogs. The trend to longer E deceleration time in female dogs only became statistically significant after indexing to √R-R. A duration and Adur/√R-R was negatively related to body weight.

B.25.1.5. *Doppler parameters of pulmonary venous flow*

A number of the Doppler parameters of PVF were influenced by the independent variables in the DCM group. Results are shown in Table B.28. (Volume II) and illustrated in the Supplement (available from the author). D and R2 wave velocities were negatively influenced by the R-R interval. S and D durations showed a positive relationship to R-R interval, and D duration was negatively influenced by advancing age in multiple linear and linear regression models. This influence of age was still significant after indexing to √R-R interval. D deceleration time was positively correlated with R-R interval, although this parameter was also significantly

influenced by gender in multiple and simple linear regression models, which was retained after indexing to √R-R; D deceleration time showed a trend to longer values in the female Newfoundlands. The R2 wave duration increased with advancing age, even after indexing to √R-R.

B.25.1.6. *Doppler parameters of right sided function*

The limited analysis of right sided function and the effect of independent variables on these parameters are shown in Table B.29. (Volume II). RPS PAv was negatively influenced by age, although no independent variable influenced PAv recorded from the LPS view in the DCM group. Tricuspid Av tended to decrease in association with lengthening R-R interval, but no other independent variable influenced any of the tricuspid inflow parameters.

B.25.2. *dFS<18% group*

B.25.2.1. *2D parameters*

The 2D echocardiographic parameters of the left ventricle showed some significant relationships with the independent variables in the dFS<18% group (Table B.30) (Volume II). LVdv was influenced by both BSA or weight (positively) and age (negatively) in multiple linear regression models. This effect of age was evident after normalising to BSA to give the EDVI, with a decline in EDVI associated with increasing age. LVsv was influenced by body weight or BSA. EF showed a significant association with increasing R-R interval. SV, determined by the single plane ellipse method, was influenced by BSA or weight as well as age (negatively) and R-R interval in a multiple linear regression model. Once indexed to BSA, the SVI shows a significant relationship with age (negative) and R-R interval (positive) in a multiple linear regression model.

The 2D echocardiographic left atrial parameters which are influenced in independent variables in the dFS<18% group are shown in Table B.30. (Volume II). LAad, LAas, LAld, LAls, 2D LAd and ratio of LAd:Aod are all positively and significantly

associated with BSA or weight. In addition, multiple linear regression models shows the negative influence of age on LAad and the short axis 2D LAd.

B.25.2.2. *M-mode parameters*

The relationships between the M-mode parameters and various independent variables are shown in Table B.31. (Volume II). LVIDd was significantly influenced by BSA or weight and also negatively by age, in the multiple linear regression model. However, after normalising for BSA, no other variable was significantly related to LVIDd. The LVIDs was positively associated with BSA and weight. IVSd was not associated with any variable, whether or not indexed to BSA. An apparently significant trend to lower IVSs thickness in female Newfoundlands was no longer evident after normalising for BSA. LVpwd and LVpws, without or with normalisation to BSA, were not significantly predicted by any of the independent variables tested. However the LVpwd/LVIDd was negatively influenced by weight or BSA. FS% and Teicholz derived EF were both influenced by R-R interval, tending to increase at slower heart rates. The ratio of mitral EPSS to LVIDd declined with increasing R-R interval. M-mode LAs was influenced by BSA or weight. Although the M-mode Aod appeared to be significantly greater in male Newfoundlands, once this was normalised to BSA (Aod/BSA), this gender influence was no longer significant. Mitral annulus motion was not influenced by any significant variables when the septal or mean parameters were considered. MAMlateral, however was significantly negatively influenced by age and positively influenced by weight or BSA in simple linear regression and multiple linear regression models.

B.25.2.3. *Doppler parameters of aortic flow*

The Doppler parameters of aortic flow were not greatly influenced by the independent variables assessed in the dFS<18% group. Results are presented in Table B.32. (Volume II). ET was influenced by age, the R-R interval and gender in a multiple linear regression model. Simple linear regression equations are also recorded for each of these parameters. ET declined with advancing age, increased with R-R interval and tended to be higher in bitches than male dogs. Once indexed to

√R-R, multiple linear regression still showed similar significant relationships of dFS<18% ET and age and gender, although significant p values were no longer obtained if simple linear regression models were applied to these variables. There was an apparently significant relationship between peak aortic velocity and the grade of aortic regurgitation as described previously in Section B.24.8.

B.25.2.4. *Doppler parameters of mitral flow*

Mitral inflow Doppler parameters were assessed for any significant predictive influence on the variables body weight or body surface area, age, gender and R-R interval for dogs in the dFS<18% group. Results are shown in Table B.33. (Volume II). The mitral E:A velocity ratio was apparently greater in male Newfoundlands although this result was not statistically significant. Evti was significantly higher in male Newfoundlands and A wave velocity normalised to total mitral vti (Av/total vti) was significantly greater in females. A duration was positively and significantly related to R-R interval.

B.25.2.5. *Doppler parameters of pulmonary venous flow*

Doppler parameters of PVF in the dFS<18% group were assessed for dependency on any of the independent variables. Results are shown in Table B.34. (Volume II). Increasing R-R interval was associated with a decrease in Arv and Sv. D deceleration time increased with lengthening R-R interval, although the other wave durations were not significantly influenced by R-R interval or any other independent variable. However, R2duration/√R-R tended to decrease with advancing age.

B.25.2.6. *Doppler parameters of right sided function*

The limited analysis of right sided function and the effect of independent variables on these parameters are shown in Table B.35. (Volume II). LPS PAv decreased with advancing age, although the influence of age or any other independent variable was not apparent on the RPS recorded pulmonary artery spectrum. Tricuspid Ev tended to decline with advancing age, although age did not significantly influence the tricuspid E:A velocity ratio.

B.25.3. *dFS18-20% Group*

B.25.3.1. *2D parameters*

The relationships between the independent variables and the two-dimensional echocardiographic parameters of the left ventricle are shown in Table B.36. (Volume II). LVdv was apparently significantly greater in male Newfoundlands, which was still significant after normalisation to BSA (EDVI). LVsv was influenced by BSA or weight. The total left ventricular stroke volume was influenced by R-R interval which was still apparent after normalisation for BSA (SVI).

The relationships between the independent variables and the two-dimensional echocardiographic parameters of the left atrium are shown in Table B.36. (Volume II). LAad was significantly influenced by BSA or weight and R-R interval in a multiple linear regression model. LAld was influenced by BSA or weight. LAls and short-axis 2D LAd were significantly greater in male Newfoundlands, although these were not normalised to BSA to check on a genuine gender influence. LAd also increased with size. 2D Aod increased with BSA or weight and age in a multiple linear regression model. The 2D ratio of LAd:Aod increased with longer R-R intervals. The LAEI significantly decreased in dogs with larger BSA or weight.

B.25.3.2. *M-mode parameters*

The associations between independent variables and M-mode parameters are given in Table B.37. (Volume II) for the dFS18-20% group of Newfoundlands. RVd was significantly associated with BSA or weight. LVIDd was also significantly predicted by BSA and weight but also R-R interval in a multiple linear regression model, which was evident even after normalisation for BSA. Similar relationships were demonstrated for LVIDs, although the predictive influence of the R-R interval was only apparent after normalisation for BSA. The interventricular septal thicknesses, in diastole or systole, whether or not indexed to BSA, showed no association with any independent variable. LVpwd and LVpws were apparently significantly thicker in male Newfoundlands, although the influence of gender was rendered insignificant

after normalisation to BSA. %thIVS increased in association with lengthening R-R interval. The M-mode Aod increased with body size and advancing age, although the influence of age was no longer apparent after indexing the parameter to BSA. MAMseptal significantly decreased with increasing R-R interval. MAMlateral was significantly influenced by body weight (positively) and negatively by age in a multiple linear regression model. No significant variables predicted MAMmean.

B.25.3.3. *Doppler parameters of aortic flow*

The Doppler parameters of aortic flow were under the influence of various independent variables in the dFS18-20% group (Table B.38. Volume II). Aov tended to decrease with lengthening R-R interval. The dv/dt_{mean} decreased with increasing R-R interval. ET and PEP were influenced by both R-R interval and age in a multiple linear regression models. Simple linear regression showed that ET significantly increased in association with R-R interval and tended to decline with advancing age, although this influence of age was no longer apparent after indexing to √R-R. PEP was also significantly positively related to the R-R interval and it tended to increase with advancing age. This influence of age on PEP was still evident after indexing to √R-R. The PEP:ET ratio and PEP:ET/√R-R also increased with age in the dFS18-20% group. Vcf was negatively significantly related to the R-R interval and Vcf increased with advancing age. Even after indexing to √R-R, this relationship between Vcf and age was retained, although gender also appeared to significantly influence Vcf (tending to be higher in female dogs) in a multiple linear regression model although Vcf/√R-R interval versus age or sex-code in simple linear regression models failed to result in significant p values.

B.25.3.4. *Doppler parameters of mitral flow*

Mitral inflow Doppler parameters for the influence of independent variables in the dFS18-20% group. Results are shown in Table B.39. (Volume II). The mitral E:A velocity ratio was significantly influenced by weight and age in a multiple linear regression model. Simple linear regression for dFS18-20% Ev:Av and weight showed an positive relationship. The trend to decreasing ratio in association with

advancing age did not achieve statistical significance in a simple linear regression model. Normalised A wave velocity (=Av/total vti) showed that both R-R interval and body weight significantly predicted this parameter in a multiple linear regression model. Simple linear regression showed that this parameter decreased with lengthening R-R interval, although simple linear regression for this Av/total vti and weight did not result in a statistically significant relationship, despite the trend to lower results with increasing weight.

The proportion of LV early filling (=Evti/total vti) showed both R-R interval and weight were significantly predictive in a multiple linear regression model. Increasing R-R interval resulted in increased Evti/total vti. The trend to increased proportion of early filling with heavier dogs was not statistically significant in a simple linear regression model. Contrasting influence of R-R interval on the proportion of late LV filling (=Avti/total vti) was apparent, with weight also negatively influencing this ratio in a multiple linear regression model.

E duration and A duration were both significantly influenced by the R-R interval, and no other independent variables were identified after indexing these parameters to √R-R. No independent variable predicted mitral E deceleration.

B.25.3.5. *Doppler parameters of pulmonary venous flow*

The effect of the independent variables on parameters of PVF are shown in Table B.40. (Volume II) for the dFS18-20% group. Arv and Sv decreased with lengthening R-R interval. D duration and D deceleration time increased with longer R-R intervals. In a multiple linear regression model, both body weight and gender significantly influenced R2 duration indexed to R-R interval, although these relationships had not been apparent for R2 wave duration prior to this. The equation showed that R2 dur/√R-R tended to increase in heavier dogs, and was greater in females than males. Simple linear regressions of R2dur/√R-R and weight or sex-code alone did not result in statistically significant p values. The S:D velocity ratio was negatively influenced by lengthening R-R intervals.

B.25.3.6. *Doppler parameters of right sided function*

The limited analysis of right sided function and the effect of independent variables on these parameters are shown in Table B.41. (Volume II). RPS PAv was negatively associated with age. No independent variable significantly influenced any of the parameters of tricuspid inflow.

B.25.4. *LVE group*

B.25.4.1. *2D parameters*

The two-dimensional echocardiographic parameters of the LV which were influenced by independent variables are shown in Table B.42. (Volume II). No independent variable was significantly predictive of the LV diastolic volume or the EDVI. The LV systolic volume and ESVI were positively influenced by the R-R interval. The ejection fraction calculated from the single plane ellipse method was negatively associated with age.

The two-dimensional echocardiographic parameters of the LA which are influenced by various independent variables are detailed in Table B.42. (Volume II). No independent variable was predictive of any of the four chamber view left atrial parameters in diastole or systole. The 2D short axis view of the diastolic left atrium (LAd) and the 2D ratio LAd:Aod showed significant negative associations with age.

B.25.4.2. *M-mode parameters*

The M-mode echocardiographic parameters which were influenced by various independent variables are shown in Table B.43. (Volume II). LVIDd was influenced both by BSA or weight and also gender, although it should be noted that male and female Newfoundlands included in this group were selected by gender specific criteria. Once normalised to BSA, there was no longer any gender influence apparent on the LVIDd/BSA parameter. LVIDs was also positively influenced by BSA or weight. IVSd indexed to BSA showed a negative influence of R-R interval. The

LVpwd indexed to BSA showed higher values in female than male Newfoundlands. The LVpws indexed to BSA declined in association with advancing age and %thLVpw also paralleled this age association. M-mode Aod declined with increasing BSA or weight. MAMseptal was positively influenced by both R-R interval and body weight or BSA in multiple linear regression models. However, the relationships of MAMseptal and either of these independent variables were not statistically significant in simple linear regression models. No independent variables were identified which significantly predicted MAMlateral or MAMmean.

B.25.4.3. *Doppler parameters of aortic flow*

The Doppler parameters of aortic flow were also assessed for dependency on independent variables in the LVE group. Results are shown in Table B.44. (Volume II). Aortic flow peak acceleration (dv/dt_{max}) was significantly predicted by both age (tending to decline with advancing age) and gender (higher in male dogs) in a multiple linear regression model, although simple linear regression of LVE dv/dt_{max} versus age or gender alone did not result in significant p values. The aortic flow acceleration time was also positively influenced by age, although this relationship was no longer significant after indexing to √R-R. PEP also apparently was significantly influenced by age, tending to increase in older dogs, although this relationship was also no longer significant after indexing to √R-R interval. The PEP:ET ratio appeared to increase in association with advancing age, although age was not a significant predictor once with parameter was normalised for heart rate by dividing by √R-R interval.

B.25.4.4. *Doppler parameters of mitral flow*

The Doppler parameters of mitral inflow which were influenced by some of the independent variables are shown in Table B.45. (Volume II). The mitral E:A velocity ratio was positively influenced by both body weight and R-R interval in a multiple linear regression model, although neither parameter on its own resulted in a statistically significant simple linear regression. No other mitral inflow parameters were significantly predicted by any other independent variable.

B.25.4.5. *Doppler parameters of pulmonary venous flow*

PVF Doppler parameters were also assessed for the predictive influences of the independent variables. Results are shown in Table B.46. (Volume II). Dv significantly increased in heavier dogs. D deceleration time tended to increase with advancing age, a relationship which became even more significant after indexing to √R-R interval. The S:D velocity ratio decreased with lengthening R-R interval.

B.25.4.6. *Doppler parameters of right sided function*

The limited analysis of right sided function and the effect of independent variables on these parameters were assessed (Table B.47. Volume II). No independent variable significantly influenced any of the parameters of right sided function in the LVE group.

B.25.5. *SAS group*

B.25.5.1. *2D parameters*

The two-dimensional echocardiographic LV parameters which were influenced by independent variables are shown in Table B.48. (Volume II). LVdv was positively related to BSA or weight. LVsv was shown to be positively influenced by BSA or weight, but a multiple linear regression model also showed that the R-R interval was a positive predictor and age was a negative predictor of LVsv. The ESVI retained this association with age and R-R interval in a multiple linear regression model.

The two-dimensional echocardiographic left atrial parameters which were influenced by independent variables are shown in Table B.48. (Volume II). LAad was positively influenced by BSA or weight and R-R interval in a multiple linear regression model. LAld was also influenced by R-R interval. LAas and LAls were both influenced solely by BSA or weight.

B.25.5.2. *M-mode parameters*

M-mode echocardiographic parameters which were influenced by independent variables in Newfoundlands in the SAS group are shown in Table B.49. (Volume II). RVd was negatively associated with the R-R interval. LVIDd was positively influenced by the R-R interval although this association was no longer significant after indexing to BSA. LVIDs was associated with BSA or weight. The interventricular septum and left ventricular posterior wall measurements in systole or diastole or indexed to BSA showed no evidence that they could be predicted by the independent variables. The LVpwd:LVIDd ratio showed a significant trend to increasing with age. FS and EF (Teicholz method) both declined with increasing body weight. M-mode Aod indexed to BSA was greater in female Newfoundlands. MAMseptal was significantly positively influenced by R-R interval. No significant independent variables were identified to influence MAMlateral. MAMmean was negatively influenced by age in a multiple linear regression model, and positively influenced by R-R interval.

B.25.5.3. *Doppler parameters of aortic flow*

The Doppler parameters of aortic flow were assessed for the predictive influence of various independent variables on the results in dogs with aortic velocity exceeding 1.7 m/s (the SAS group). Results are shown in Table B.50. (Volume II). No independent variable influenced aortic velocity, velocity time integral or peak or mean flow accelerations. The acceleration time was apparently longer in male dogs, although this relationship was no longer significant after indexing to √R-R interval. PEP and ET increased in association with the R-R interval, although after indexing to √R-R, no other independent variable influenced these parameters. Vcf/√R-R was negatively related to advancing age, although the influence of age was not statistically significant for uncorrected Vcf.

A weak positive but significant relationship was identified by linear regression between the grade of aortic insufficiency and the peak aortic velocity, as recorded in Section B.24.8.

B.25.5.4. *Doppler parameters of mitral flow*

The Doppler parameters of mitral inflow were assessed for the significant influence of any of the independent variables such as body weight or BSA, age, gender or R-R interval on the parameters. The results are shown in Table B.51. (Volume II). Mitral Av and Av/total vti tended to decline in association with lengthening R-R interval, and the E:A velocity ratio was positively related to the R-R interval. Evti and the E:A vti ratio were also positively predicted by R-R interval. In a multiple linear regression model as well as simple linear regression, weight was identified as another positive predictor of mitral E:A vti ratio. Body weight was significantly positively associated with the proportion of early LV filling (=Evti/total vti) and negatively associated with the proportion of late LV filling (=Avti/total vti). Age was a negative predictor of E:A velocity ratio in the multiple linear regression model with R-R interval, although this was not a statistically significant relationship in a simple linear regression model. However, an age related decline of mitral E wave velocity normalised to total vti (Ev/total vti). Significant trends to increasing E duration and deceleration time were evident with advancing age, although these relationships were no longer significant after indexing to √R-R interval. A duration was positively related to R-R interval and also age. The predictive influence of age was still apparent after indexing A duration to √R-R interval.

B.25.5.5. *Doppler parameters of pulmonary venous flow*

The Doppler parameters of PVF were also assessed for the predictive influence of the independent variables in the SAS group. Results are shown in Table B.52. (Volume II). Ar duration decreased with lengthening R-R interval. Dv and Dvti were significantly negatively influenced by body weight. Dvti was also influenced by gender in a multiple linear regression model (higher values in male dogs), although this relationship was not statistically significant in a simple linear regression model. S duration and Sdur/√R-R were significantly positively associated with body weight. D duration and D deceleration time were positively correlated with R-R interval.

B.25.5.6. *Doppler parameters of right sided function*

The limited analysis of right sided function and the effect of independent variables on these parameters in the SAS group are shown in Table B.53. (Volume II). LPS PAv was negatively associated with age. A significant relationship was identified between peak pulmonic velocity and peak aortic velocity in the SAS group, which was described previously (Section B.24.11.1.).

Tricuspid Av decreased with lengthening R-R interval and the tricuspid E:A velocity ratio was significantly influenced by both R-R interval and age in a multiple linear regression model. The positive relationship between E:A ratio and R-R interval was still significant in a simple linear regression model, although the trend to negative predictive influence of advancing age on this ratio did not achieve statistical significance in a simple linear regression model.

B.26. *Serial scans in the Newfoundland population*

Details from the Newfoundland individuals concluded to be Normal after two or more scans at the time of the second or final scan are shown in Tables B.54.a. - B.54.h. (Volume II). Dogs with DCM are detailed in Tables B.55.a. - B.55.h. (Volume II). Dogs with depressed fractional shortening, both dFS<18% and dFS18-20%, and the single individual in the LVE category, are shown in Tables B.56.a. - B.56.h. (Volume II). Individuals with a final diagnosis with an aortic velocity exceeding 1.7 m/s are detailed in Tables B.57.a. - B.57.h. (Volume II).

The general details regarding each individual and each scan are given in Tables B.54.a. - B.57.a. Two-dimensional echocardiographic left ventricular parameters are shown in Tables B.54.b. - B.57.b., left atrial parameters in Tables B.54.c. - B.57.c. and M-mode echocardiographic parameters in Tables B.54.d. - B.57.d. Doppler parameters of aortic flow are displayed in Tables B.54.e. - B.57.e., mitral flow in Tables B.54.f. - B.57.f., pulmonary venous flow in Tables B.54.g. - B.57.g. and right sided Doppler parameters in Tables B.54.h. - B.57.h. Means and standard deviations (sd) are recorded.

These tables include the results of the percentage difference between the means (%diffMeans) and the standard deviation expressed as a percentage of the mean (sd%Mean) for each echocardiographic parameter. Where %diffMeans was greater than sd%Mean, this was indicated by displaying the %diffMean value in bold type. If the %diffMeans was a negative value, this indicates that the later scan has a larger value than the initial scan, and where this value is positive, the later scan is smaller. When comparing %diffMeans with sd%Mean, the positivity or negativity was ignored. The final set of results displayed in these tables was the significance level of the Student *t* test result after this test had been applied to the raw data sets from the initial and second (or final) scans for each echocardiographic parameter.

B.26.1. *The Normal Category*

There were eleven dogs in this category.

B.26.1.1. *2D LV parameters*

Results are shown in Table B.54.b. LVdv %diffMeans ranged from 1.01 to 58.08%. From the repeatability study, the coefficient of variation (c.o.v.) for the pooled data was 17.8% (data not shown; available in the Supplement, obtainable from the author). Three out of the eleven dogs had %diffMeans which exceeded this figure. LVsv %diffMeans ranged from -1.34% to 28.73%. The c.o.v. identified in the repeatability study was 24.14%. Only two values exceeded this. EF %diffMeans ranged from 0.42 - 52.08%, compared with c.o.v. of 16.79%. Three individuals exceeded this value. The SV %diffMeans ranged from -1.95% to -155.33%. Three individuals exceeded the c.o.v. of 24.83% for SV.

B.26.1.2. *2D LA parameters*

Results are shown in Table B.54.c. LAad %diffMeans ranged from 4.21% to -30.22%, with four individuals with values exceeding the c.o.v. of 15.96%. LAas %diffMeans ranged from -1.70% to -35.10%, with six dogs with values >15.66% (c.o.v.). LAld %diffMeans ranged from 2.78 to -24.23%, with one dog >c.o.v. of 13.47%. LAls %diffMeans ranged from -1.94 to -19.08%, with three dogs >c.o.v. of 8.16%. From the short axis view, LAd %diffMeans ranged from -0.23 to -22.06%, with six dogs >c.o.v. value of 10.20%. Aod %diffMeans ranged from -0.27 to -24.67%, with differences in two dogs greater than c.o.v. of 10.71%.

B.26.1.3. *M-mode parameters*

Results are shown in Table B.54.d. (a) and B.54.d. (b). RVd %diffMeans ranged from 11.39 to -168.76%, with six dogs >c.o.v. of 27.37%. The %diffMeans for IVSd and IVSs ranged from 1.08 to -19.59% and 1.82 to -35.35% respectively, with three dogs >c.o.v. of 17.71% (IVSd) and two dogs >c.o.v. of 15.96% (IVSs). The %diffMeans for LVpwd and LVpws ranged from 0% to -23.49% and 0.77% to -24.36%, with six dogs >c.o.v. of 14.63 (LVpwd) and five dogs >c.o.v. of 12.77

(LVpws); the same five dogs in the LVpws were also in the LVpwd group. LVIDd %diffMeans ranged from 0.45% to 15.31%, with five dogs greater than c.o.v. of 7.74%. The %diffMeans for LVIDs ranged from -1.66 to 18.98%, with four dogs exceeding the c.o.v. of 10.43%. FS% %diffMeans ranged from 0.75% to -45.99%, with five dogs greater than c.o.v. of 17.57%. Mitral EPSS %diffMeans ranged from 6.83% to -92.49%, with five dogs with values >c.o.v. of 26.23%. M-mode Aod and LAs ranged from -0.95% to -10.88% (one dog with differences >c.o.v. of 10.79%) and 1.64% to 24.04% (three dogs with values >c.o.v. of 13.42%) respectively.

MAM septal and MAM lateral %diffMeans ranged from 1.3% to -49.73% and 4.57% to -34.68% respectively, with four dogs and five dogs exceeding the coefficients of variation of 21.57% (MAMseptal) and 18.79% (MAMlateral) respectively.

B.26.1.4. *Doppler parameters of aortic flow*

Results are shown in Table B.54.e. The %diffMeans for Aov ranged from 6.25% to 27.87%, with one dog with differences greater than the c.o.v. of 19.39%, although in a number of individuals, subcostal measurements were not obtained in both scans. Aov (L.Ap.view) showed a range in %diffMeans of 3.82% to 27.62%, with three dogs >c.o.v. of 20.86%. Other comparisons were generated from the subcostal view of aortic flow. The %diffMeans for aortic vti ranged from 3.45% to 35.26% with the same dog with a difference >c.o.v. of 21.05%. When dv/dt_{max} and dv/dt_{mean} were considered, differences ranged from 3.98% to 46.11% and 13.25% to 34.68% respectively, with the same single dog with differences exceeding the c.o.v. values of 24.69% (dv/dt_{max}) and 26.08% (dv/dt_{mean}). The systolic time interval (STI) data were obtained from the subcostal data, where available, but included left apical aortic flow data also. The respective %diffMeans for PEP (uncorrected) and ET ranged from 3.74% to -14.95% (with four dogs with differences > PEP (uncorrected) c.o.v. of 7.84%) and -2.89% and 14.36% (with three dogs > ET c.o.v. of 6.70%). The %diffMeans for the corrected PEP:ET ratio ranged from 2.30% to -43.77%, with four dogs with values greater than the reported c.o.v. of 10.63% (although this was generated from uncorrected data). Vcf %diffMeans ranged from 2.09% to 29.18%,

although had not been assessed in the repeatability study. The acceleration time %diffMeans ranged from 8.02% to -16.94%, with no dogs exceeding the c.o.v. value of 20.00%.

B.26.1.5. *Doppler parameters of mitral flow*

Results are shown in Table B.54.f. In the Normal Newfoundland group, the %diffMeans for mitral E and A wave velocities ranged from -0.13% to 33.98% and -1.76% to -24.29% respectively. The differences exceeded the c.o.v. for Ev (22.22%) and Av (22.92%) in two cases. Evti and Avti %diffMeans ranged from 2.22% to 32.17% (two dogs exceeding c.o.v. of 16.67%) and -1.85% to -69.23% (with three dogs exceeding c.o.v. of 40.91%), respectively. The %diffMeans for E duration, E deceleration time and A duration ranged from -1.33% to -27.01%, -4.91% to 36.95% and -0.51% to -22.75% respectively. E duration c.o.v. of 13.48% was exceeded in five dogs, E deceleration time (24.27%) in two cases and A duration c.o.v. (16.48%) was exceeded in four cases. IVRT %diffMeans ranged from 0.29% to 18.92% and no dogs exceeded the c.o.v. value of 25.45%.

B.26.1.6. *Doppler parameters of pulmonary venous flow*

Results are shown in Table B.54.g. The %diffMeans S and D velocities ranged from -1.17% to 36.59% and -2.46% to 25.11% respectively. The c.o.v. values were 16.22% and 17.65% respectively, and these were exceeded in two cases for each parameter (only one dog being the same). PVF Ar velocity %diffMeans ranged from 2.01% to -66.15%, with two dogs with differences >c.o.v. value of 46.43%. R2 velocity differences ranged from 0.69% to -108.75%, with the c.o.v. value of 33.33% exceeded in five cases. Svti and Dvti %diffMeans ranged from -2.50% and 40.43% (>c.o.v. value of 21.67% in four dogs) and -4.26% to -48.65% (>c.o.v. of 21.78% in four dogs) respectively. Ar duration %diffMeans ranged from -4.21% to 38.13% with three dogs >c.o.v. value of 19.18%. S duration %diffMeans ranged from 2.46% to 16.01% with two dogs >c.o.v. value of 12.99%. D duration and D deceleration time ranged from 0.12% to -24.04% and 3.53% to 26.03%, respectively with three dogs

>c.o.v. of 17.92% (D duration) and no dogs exceeding c.o.v. of 26.16% (D deceleration time).

B.26.1.7. *Right sided Doppler parameters*

Results are shown in Table B.54.h. The RPS PAv %diffMeans ranged between 1.76% and 48.40%, five dogs with differences >c.o.v. for RPS PAv of 13.33%. LPS PAv %diffMeans ranged from 1.24% to -46.08%, with four cases >c.o.v. value of 17.48%. Tricuspid E and A wave velocities %diffMeans ranged from 4.62% to 23.95% (one dog >c.o.v. of 22.95%) and -5.21% to 37.91% (one dog >c.o.v. of 35.29%), respectively.

B.26.2. *The Dilated Cardiomyopathy (DCM) category*

Twelve dogs had a final diagnosis of DCM during serial evaluation. Results are shown in Tables B.55.a. - B.55.h. The general details, including the category under which each scan was collated and the time interval between scans, was recorded in Table B.55.a. The status and any comments about the individuals were also recorded. One dog (NF002/P500) was symptomatic in atrial fibrillation at the time of the initial scan, while untreated. Initially, she was prescribed an ACE inhibitor, but, with continued deterioration, she required standard medication with enalapril, frusemide and digoxin, and subsequently improved. Dogs NF003/P505 and NF042/P42 remained with occult disease, and both received an ACE inhibitor. NF008/P534 suddenly developed severe congestive heart failure. He received an ACE inhibitor after the time of his second scan, when the owner reported breathlessness, but with continued absence of symptoms, the owner stopped treatment and signs developed six weeks later. NF056/P262 did not receive any medication prior to clinical signs developing at the time of the second scan. One dog with LVE progressed to DCM, but was euthanased due to pulmonary neoplasia (NF007/P215). There were four dogs initially included in one of the dFS categories who progressed to DCM (NF031/P536, NF033/P531, NF010/P51 & NF070/P274). Two dogs had initially been classified as "normal" (NF036/P40 & NF029/P723), but later developed evidence of occult DCM.

B.26.2.1. *2D Parameters*

If an arbitrary significant difference was designated as >20% between scans, the following 2D LV parameter results were obtained from the DCM group (Table B.55.b.). LVdv significantly increased in four dogs. LVsv significantly increased in two dogs, and decreased in the one dog (NF002/P500) who showed a response to treatment. EF significantly decreased in one dog and increased in five dogs. Left atrial parameters are shown in Table B.55.c. LAad significantly increased in three dogs and decreased in one dog. LAas significantly increased in three dogs. LAld significantly increased in two dogs and decreased in one dog. LAls increased in two dogs. 2D LAd significantly increased in four dogs. No significant differences were identified for Aod.

B.26.2.2. *M-mode parameters*

From the M-mode results from the DCM group (Tables B.55.d.(a) & B.55.d.(b)), significant differences were recorded for the RVd, with decreases in RVd of >20% in five dogs and increases in RVd of >20% in two dogs. IVSd significantly increased in three dogs and IVSs significantly increased in one dog. LVpwd significantly increased in three dogs and LVpws significantly increased in one dog. LVIDd and LVIDs both significantly increased in two dogs. FS% decreased by >20% in two dogs and increased by >20% in four dogs. Mitral EPSS increased by >20% in four dogs and decreased by >20% in two dogs. M-mode LAs significantly decreased in three dogs and increased in one dog. No significant differences were identified for the Aod. MAMseptal was significantly increased by >20% in three dogs. MAMlateral was significantly increased in one dog and decreased in two dogs.

B.26.2.3. *Doppler parameters*

Doppler parameters of aortic flow are shown in Table B.55.e. The peak Aov (subcostal view) was increased by >20% in two dogs and not significantly decreased in any, although there was a trend to decreasing velocities. One dog showed a significant increase in aortic vti, and two dogs showed a significant decrease in vti.

The dv/dt_{max} was significantly decreased in one dog, and significantly increased in one dog. The dv/dt_{mean} was significantly decreased by >20% in four dogs and increased in one. The acceleration time was significantly longer in one dog. The corrected PEP was significantly (>20%) longer in four dogs but was shorter in one dog (NF042/P542). ET was significantly shorter in one dog (NF008/P534). The corrected PEP:ET ratio was significantly greater in five dogs and reduced in one dog (NF042/P542). Vcf was significantly decreased in three dogs and increased in three dogs (but was, however, less than 1.00 circs/s in all three and therefore was abnormal).

Doppler parameters of mitral inflow for the DCM group are shown in Table B.55.f. Mitral Ev was different (>20%) in two dogs (increased) and two dogs (decreased) and Av was increased in two dogs and decreased in one dog. Evti was significantly increased in two dogs and decreased in one dog. Avti was significantly increased in two dogs and decreased in seven dogs. E duration was significantly shorter in one dog and was longer in one dog. E deceleration time was significantly shorter in three dogs and longer in one dog. No differences exceeding 20% were identified for mitral A wave duration. IVRT was greater by >20% in two dogs and was significantly shorter in three dogs.

The PVF parameters for the DCM group are shown in Figure B.55.g. Arv was increased (>20%) in three dogs and decreased in one dog. Sv was significantly increased in one dog and decreased in four dogs. Dv was increased >20% in four dogs and decreased in one dog. R2v was significantly increased in two dogs and decreased in two dogs. Svti increase (>20%) was identified in one dog, but a significant decrease was recorded in four dogs. Dvti was increased in two dogs and decreased in one dog. Ar duration was significantly longer in four dogs. S duration was significantly shorter at the second scan in one dog. D duration was significantly longer in two dogs and shorter in two dogs. D deceleration time was significantly longer in two dogs and shorter in three dogs.

Doppler parameters for the right heart are shown in Table B.55.h. for the DCM group. RPS PAv was significantly lower (>20%) in three dogs. LPS PAv was significantly higher in one dog. Tricuspid Ev was significantly greater in three dogs and lower in four dogs. Tricuspid Av was significantly greater (>20%) in two dogs and lower in five dogs.

B.26.3. *The depressed fractional shortening (dFS) category*

There were nineteen dogs in the dFS category. General details, including classification of each serial scan, are recorded in Table B.56.a. Six dogs moved from Normal to dFS18-20%. One dog moved from dFS18-20% to "normal", although the presence of VPCs suggested otherwise, which warranted inclusion of this individual in the "equivocal" category (NF107/P289). Three dogs changed from "normal" to dFS<18%. Nine dogs remained in one or other of the dFS categories (either dFS<18% or dFS18-20%) for both scans.

B.26.3.1. *2D parameters*

The 2D LV parameters are shown in Table B.56.b. and the LA parameters in Table B.56.c. In the dFS group, the LVdv was increased by >20% in three dogs and decreased by >20% in two dogs. LVsv was significantly increased in one dog and was decreased in two dogs. EF was significantly (>20%) increased in four dogs and decreased in three dogs. LAad was significantly increased in two dogs and decreased in two dogs. LAas was significantly increased in one dog and decreased in two dogs. LAld did not significantly change in any dog. LAls was significantly increased in two dogs. 2D LAd was significantly greater in two dogs. No significant changes in Aod were identified.

B.26.3.2. *M-mode parameters*

M-mode parameters are shown for the dFS category in Tables B.56.d.(a) and B.56.d.(b). The M-mode criteria for the dFS group showed a number of significant differences for RVd, with seven dogs with values >20% above baseline and six dogs with values of 20% less than baseline. There was one individual with a difference

smaller than 20% for the IVSd measurement, and no differences in either direction for IVSs of >20%. LVpwd was not significantly different between scans for any individual in the dFS category, although two individuals showed significantly greater LVpws from the initial scan. LVIDd was only significantly different in one individual (NF037/P48), when it was dramatically smaller (54.58 mm to 41.68 mm). LVIDs was significantly increased in one individual (NF049/P287). FS% was decreased (>20%) in eight individuals and increased in three dogs. Mitral EPSS was significantly increased in five dogs but decreased in four dogs. M-mode LAs was increased by >20% in one dog and decreased in one dog. Aod increased by >20% in three dogs. MAMseptal was significantly greater in three dogs and was significantly reduced in two dogs. MAMlateral showed differences >20% in one dog (decrease).

B.26.3.3. *Doppler parameters*

Doppler parameters of aortic flow are shown in Table B.56.e. Subcostal Aov was significantly decreased between scans in one individual. Aortic vti was significantly decreased in two dogs. The dv/dt_{max} was significantly (>20%) increased in three dogs and decreased in two dogs, whereas dv/dt_{mean} was significantly decreased in three dogs (two of whom were the same individuals as the significant dv/dt_{max} decreases). Acceleration time was significantly longer in one dog and was shorter in three dogs. The corrected PEP was significantly longer in three dogs. There were no significant differences in ET between scans for any individual. The corrected PEP:ET ratio was significantly longer in five dogs, with a trend to this ratio increasing in most dogs in this category. Vcf was significantly greater in four individuals and significantly reduced by >20% in seven dogs.

Doppler parameters of mitral inflow are recorded in Table B.56.f. Mitral Ev was significantly (>20%) increased in two dogs and decreased in three dogs. Av %diffMeans exceeded 20% in four dogs (increased) and three dogs (decreased). Evti was significantly greater in three dogs and less in five dogs. Avti was significantly greater on repeat scan in eight dogs and was smaller in five dogs. E duration was significantly (>20%) increased between scans in three dogs and decreased in two

dogs. E deceleration time was significantly different in three dogs (increased; the same dogs as E duration increases) and three dogs (decreased). A duration was significantly increased in four dogs and decreased in one dog. IVRT was significantly longer in one dog and was significantly shorter in three dogs.

Doppler parameters of PVF for the dFS group are detailed in Table B.56.g. Arv was significantly increased by >20% in four dogs and was decreased in one dog. Sv was significantly increased in three dogs and decreased in two dogs. Dv was significantly increased in two dogs, and decreased in one dog. R2v was increased (>20%) in four dogs and decreased (>20%) in five dogs. Svti was significantly greater in three dogs and less in four dogs. Dvti was greater in one dog and less in six dogs. Ar duration increased in three dogs and decreased in two dogs. S duration was significantly longer in one dog and shorter in two dogs. D duration and deceleration time was significantly longer in one dog; D duration was significantly shorter in three dogs and D deceleration time significantly decreased in five dogs.

Doppler parameters of right sided results are detailed in Table B.56.h. RPS PAv was decreased (>20%) in four dogs. LPS PAv was significantly different in two dogs (greater) and seven dogs (decreased). Tricuspid Ev was significantly increased between scans in three individuals and decreased in three. Tricuspid Av was significantly increased (>20%) in eight dogs and decreased in two dogs.

B.26.4. *Left ventricular enlargement (LVE) category*

Only one individual had a repeat scan which remained in this category on both occasions (NF021/P552). Results are shown in Table B.56.a. - B.56.h. There was no significant difference for any of the 2D LV parameters for this individual, despite a 10.61% increase in LV systolic volume. A significant increase in LAad was identified by the second scan. No significant differences for most of the M-mode criteria were reported between scans. However, FS% significantly decreased from 31.67 to 23.89% and mitral EPSS was significantly increased in the second scan. There was an apparent decrease (>20%) M-mode LAs. MAM septal was decreased

by more than 20% in the second scan. When the Doppler parameters of aortic flow where compared, dv/dt_{max} and dv/dt_{mean} significantly decreased and the acceleration time of aortic flow increased. Vcf decreased. Changes were consistent with deteriorating systolic function. Mitral inflow showed a significantly greater Avti and significantly shorter IVRT on repeat scan. PVF Ar, S, and R2 wave velocities were significantly decreased, as were the Svti and Dvti. S duration was also significantly reduced (without a major difference in heart rate between the two scans). Tricuspid Ev and Av were significantly lower than the initial scan.

B.26.5. *Category with subcostal aortic velocity >1.7 m/s (SAS category)*

Eleven dogs with an aortic velocity exceeding 1.7 m/s on at least one occasion had a minimum of one repeat scan, and NF005/P515 had two repeat scans. General information is displayed in Table B.57.a. Only three of these dogs remained in the Aov >1.7m/s group for both scans (NF055/P258, NF068/P304 & NF023/P248). NF005/P515 had the two initial scans in this category, but not the final scan. Five dogs changed between the normal and Aov>1.7m/s categories, and one dog was initially in the dFS18-20% category before the elevated aortic velocity was identified (NF091/P47).

B.26.5.1. *2D parameters*

In the SAS group, the 2D LV parameters are displayed in Table B.57.b. Significant differences, exceeding 20%, were identified in of LVdv in three dogs (increased) and one dog (decrease). LVsv was significantly increased on repeat scan in two dogs, and decreased in three dogs. EF was significantly greater in five dogs on repeat scan. Left atrial 2D parameters are displayed in Figure B.57.c. One dog had a significantly increased LAad on the second scan. One dog showed a significant decrease in LAld. LAas was significantly increased in three dogs LAls was significantly increased in two dogs. 2D LAd was significantly increased compared with initial scan in two dogs, but there were no significant differences recorded for Aod.

B.26.5.2. *M-mode parameters*

The M-mode parameters for the SAS group are recorded in Table B.57.d.(a) and B.57.d.(b). Differences exceeding 20% were identified for the RVd, with five dogs showing a significant increase and one dog showing a decrease. One dog showed a significant increase in IVSd, and another individual showed a significant increase in IVSs, with IVSs significantly decreased in one dog. LVpwd and LVpws showed no significant differences. LVIDd and LVIDs were also not significantly different between scans. FS% significantly increased in two individuals (NF005/P515 & NF058/P256). Mitral EPSS was significant increased in three dogs and decreased in two dogs. M-mode LAs was significantly decreased in one dog, with no significant differences for Aod. Septal MAM was significantly (>20%) increased in two dogs and decreased in two dogs. Lateral MAM was increased in two dogs and decreased in three dogs.

B.26.5.3. *Doppler parameters*

The Doppler parameters of aortic flow are shown in Table B.57.e. Significant differences (>20%) for Aov were identified in one dog (increase) and one dog (decrease). Significant differences in aortic vti were identified in the same individuals. The dv/dt_{max} was significantly increased in three dogs and decreased in three dogs. The dv/dt_{mean} was significantly increased in one dog and decreased in four dogs. The acceleration time was significantly increased in one dog and decreased in one dog. The corrected PEP was significantly longer (>20%) in three dogs. There were no significant differences between scans for ET or PEP:ET ratio. Vcf was significantly different, with four dogs showing an increase and one dog showing a decrease.

The Doppler parameters for mitral flow are shown in Table B.57.f. Mitral Ev was significantly (>20%) greater in two dogs and less in one dog. Av was significantly increased in two dogs and decreased in two dogs. Evti was significantly increased in three dogs. Avti was significantly increased in five dogs and decreased in one dog. E duration was significantly (>20%) increased above baseline in one dog and E

deceleration time was increased in one dog and decreased in one dog. A duration was significantly longer in five dogs. IVRT was significantly longer in two dogs and shorter in two dogs.

The PVF parameters are recorded in Table B.57.g. Arv was significantly increased in six dogs. Sv was significantly decreased in two dogs. Dv was significantly increased in one dog and decreased in two dogs. R2v was significantly larger in five dogs and was decreased in one dog. Svti was significantly increased in one dog and decreased in two dogs. Dvti vti was significantly increased in three dogs. Ar duration was significantly longer in five dogs, S duration was significantly longer in two dogs and D duration was significantly longer in three dogs and shorter in one dog. D wave deceleration time was significantly shorter in the second scan in three individuals.

The results of the Doppler parameters from the right heart analysis are shown in Table B.57.h. RPS PAv exceeded 20% difference between scans in two dogs, one with a significant increase and one with a significant decrease. LPS PAv was significantly decreased in three dogs. Tricuspid Ev was significantly increased in two dogs and decreased in one dog. Tricuspid Av was significantly increased in six dogs and decreased in three dogs.

DISCUSSION

B.27. *Beat to beat variation and the effect of respiration*

In humans, images for quantification are recorded during an end-expiratory apnoeic phase, by instructing breath-holding in patients. Dubrey and others (1997) showed that an average of four beats of regular sinus rhythm and thirteen beats of atrial fibrillation were required to estimate cardiac output with variability less than two percent. In human patients, there is usually less than 10% variation in R-R interval through an examination, which results in 33% difference in beat to beat evaluation of left ventricular volumetric indices (Seliem *et al* 1996). Breath-holding did not reduce the amount of beat to beat variability. In younger patients, with more sinus arrhythmia, these authors recommended that more than five beats should be measured. Bett and Dryburgh (1981) also reported that five or more cardiac cycles should be measured to reduce the effect of beat to beat variation for M-mode dimensions.

In this study, six measurements of each parameter were normally made. Newfoundlands do not normally have accentuated sinus arrhythmia but it does exist to a greater degree than in man. It can therefore be argued that more cardiac cycles should have been measured, and this is certainly true for dogs in atrial fibrillation, where a maximal of ten beats were measured, but typically six or seven. Most veterinary echocardiographic studies report only three measurements of each parameter. It is virtually impossible to control respiration in canine patients. Respiration, by causing rapid chest wall motion, means that is difficult to maintain transducer orientation through a consistent plane (Schiller & Foster 1996), and this was certainly a problem during M-mode examinations. Panting made it virtually impossible to record diagnostic sub-costal aortic spectra. Even with calm respiration, considerable beat to beat variation was evident in the spectra, apparently associated

with the respiratory cycle, due to altering position of the Doppler plane with blood flow.

B.28. *Normal Newfoundlands*

In generating reference ranges, this study may be criticised for including younger dogs as well as dogs over eight years old, since the younger dogs may develop DCM in the future. However, it was decided to combine the two Normal groups as there were so few animals present in the older group. Another compromise made in this study was the fact that most animals in the Normal group did not have a repeat scan to confirm normality. There were too few serially scanned dogs present in the Normal category on two or more occasions and it was felt that the group would be too small to generate statistically significant reference ranges for the breed.

In combining the two normal groups with animals over and younger than eight years of age, it was found that most parameters were not statistically significantly different between the two groups. This was true for all the M-mode parameters, although there were significant differences for some of the two-dimensional parameters, particularly left ventricular diastolic area and volume, which was significantly smaller in the older age group, as was ejection fraction and stroke volume. It was speculated that this may be an effect of age, and this was investigated further.

B.28.1. *Effect of body weight or body surface area*

In the Normal group, the relationship between body weight and BSA was linear, probably due to the fact that a single breed of approximately uniform size was being investigated. Where regression analysis identified weight or BSA as an independent variable significantly affecting 2D echo or M-mode parameters, in general, there was no advantage of using BSA over weight, except for IVSd where the relationship to body weight did not achieve statistical significance despite a significant relationship to BSA.

The M-mode LV parameters which were influenced by weight or BSA were RVd, IVSd, LVpwd, LVpws and LVIDd. IVSs and LVIDs were not influenced by weight or BSA in the Normal group, and FS or EF (Teicholz) also failed to show a significant relationship to size. Mitral EPSS showed a significant relationship to size, which was suggested by Kirberger (1991a), who found a trend to increasing EPSS for larger dogs although this did not achieve statistical significance in his study. In children, if EPSS was normalised to LVIDd, no relationship was evident with BSA, height, weight or age (Engle *et al* 1983). There was a significant relationship between size and M-mode Aod and LAs, although the ratio (LAs:Aod) was not influenced by any of the independent variables. Mitral annulus motion (MAM) was not significantly related to weight or BSA, although the apparent gender effect on septal MAM was lost once the parameter was indexed to BSA.

The 2D echocardiographic left ventricular parameters, LVld, LVdv, LVsv and SV were all significantly influenced by body weight or BSA. The parameters of left atrial size measured from the four chamber long axis view also showed similar positive relationships, and, from the short axis view, 2D Aod and LAd also showed increasing size in larger dogs, although the ratio (2D LAd:Aod) was not determined by body size.

Wong and others (1995) reported that in man, BSA was the strongest positive predictor of left ventricular volumes, followed by total skin fold thickness, which had negative associations with end-diastolic and end-systolic left ventricular volumes, even after correcting for body surface area. This indicates that the known association of body size and volumes may be counteracted in part by the relative amount of body fat, indicated by total skin fold thickness. These authors suggest that obesity is associated with lower absolute blood volume and myocardial and peripheral perfusion than normal individuals. Newfoundlands are large dogs, and a massive build is considered desirable. Although body condition was not semi-

quantitatively assessed, many tended to be over-weight in the author's opinion, particularly the older dogs. It may be that this is one reason that these Newfoundlands had smaller left ventricular size than would be expected compared to breeds such as Dobermanns. It would be useful to assess body condition or even skin fold thickness in this breed during future investigation in order to ascertain this as a possible independent variable on echocardiographic parameters.

M-mode parameters regressed to body weight or BSA have been reported in the veterinary literature. Boon and colleagues (1983) gave linear regression equations for M-mode parameters and BSA for dogs or various breeds weighing between 9.8 and 28.6 kg. The regression lines given by Lombard (1984b) were to body weight and so the two studies are not directly comparable, although a slighter wider weight range was included in the Lombard study (5 - 44 kg). Although regression lines of M-mode parameters to weight are given by Bonagura and others (1985) for dogs up to 35 kg, the regression equations are not supplied. Jacobs and Mahjoob (1988b) used multiple linear regression for BSA and cardiac cycle length. Snyder and colleagues (1995) compared these three references in illustrating the differences identified in a group of greyhounds, showing that specific breeds may differ from reference values generated for all breeds. Morrison and colleagues (1992) emphasised the importance of breed specific reference ranges using regression equations showing that for some parameters, the equations differed for breeds of differing somatotypes, and some breeds did not show significant regression with body surface area (e.g. Afghan hound). Herrtage (1994) also indicated that absolute values of normal M-mode echocardiographic data were more suitable for boxers, as no statistically significant relationship between the parameters and body size was evident. In this study, Newfoundlands do have a significant relationship between most M-mode parameters and weight or BSA, which is not unexpected when the wide range of body weights are considered. Although in this study, a table of means with confidence limits have been generated for this breed, it appears that regression tables with 95% prediction intervals should be used to compare echocardiographic results of new patients with these reference values. If regression equations published by Boon *et al* (1983) or

Lombard (1984b) were used to predict Newfoundland M-mode parameters, they resulted in significant over-estimation of the observed values and regression data. Borgarelli and colleagues (1996) reported that the use of published reference regression lines in great Danes also significantly over-predicted observed data, but unfortunately, these authors did not give great Dane specific regression equations for comparison with this Newfoundland study. This over-estimation was also noted by Koch and others (1995), in a group of normal giant breeds including Newfoundlands, great Danes and Irish wolfhounds. These findings actually may not reflect somatotypic differences of a specific or a giant breed, but may just support the statistical advice that regression equations should not be extrapolated for independent variable dimensions which had not been observed in generating the line. Indeed, Pietra and others (1998) reported that the M-mode criteria for English setters, with weights similar to those generating published regression lines, did have similar values to those predicted.

O'Grady and colleagues (1986) also published regression equations for some 2D echocardiographic parameters based on clinically normal dogs from 4.5 - 30 kg. When these equations were used to predict Newfoundland measurements, again, they significantly over-estimated those values observed in the normal Newfoundland population.

When categorising Newfoundlands prior to data analysis in this study, M-mode LVIDd was considered to be enlarged if it exceeded 50 millimetres in bitches or 55 millimetres in dogs. However, these data now indicate that the LV M-mode parameters depend on weight or BSA and are independent of gender. The mean (with confidence intervals) LVIDd of 45.35mm (44.49 - 46.21mm) do not include all Normal Newfoundlands. The mean plus or minus two standard deviations ($45.35 \pm (2 \times 4.03)$) gives limits of 37.29 - 53.41 mm, which suggests that a criterion for left ventricular enlargement may be >2 standard deviations or >53.5 mm. The mean LVIDs is 34.31 mm, and if normal ranges within two standard deviations are

considered, then LVIDs should be within 28.31 and 40.31 mm. Newfoundlands with LVIDs >40.5 mm may be suspected as being abnormal. These figures of "normality" of <(mean + 2sd) correspond to LVIDd or LVIDs of <118% of the mean value. This is greater than the criteria of 112% above predicted, used to define left ventricular enlargement in human echocardiography (Baig *et al* 1998), although this is based on the predicted values using patient height, weight and age. It is therefore preferable to compare future Newfoundland values, including the individual's weight, with the regression data generated from this study.

Doppler parameters were not usually influenced by body size. However, the mitral Avti was shown to be predicted by weight or BSA in a multiple linear regression model with gender. Mitral Av has been directly related with body mass index in humans (Kangro *et al* 1996; Pai & Stoletniy 1997). Female dogs also tended to have higher Avti. Since female dogs tend to be fatter than male dogs, this result may support the fact that left ventricular compliance is reduced in obese patients (Lewis 1996). A limitation of this study was that more detailed records of body condition, such as skin fold thickness, had not been kept, so this interesting finding could not be investigated further. No effect of body weight or BSA was identified for any of the PVF parameters.

B.28.2. *Effect of gender*

Forward stepwise regression or multiple linear regression identified gender as a significant independent variable affecting a few of these parameters. ESVI was influenced by gender. The LVld was identified by multiple linear regression to be associated with both size and gender, with male dogs and larger dogs having longer ventricles. Associated with this finding was that the ratio of LVld:LVIDd (index of sphericity) was larger in males, although when the index was corrected for BSA, this gender effect was no longer apparent. The index of sphericity used in this study was simple in contrast to others proposed (St.John Sutton *et al* 1998).

The same apparent interaction of gender effects and BSA was also seen for MAMseptal; the gender effects disappeared after correcting for BSA despite the absence of a significant relationship between BSA and MAMseptal identified by simple linear regression. MAMmean was also apparently significantly influenced by gender.

It was surprising that some of the Doppler parameters were influenced by sex. The corrected PEP was weakly but significantly related to gender, with a trend to a longer time in the male dogs although normalisation for heart rate (PEP/√R-R) suggests that gender is not a significant predictor of this parameter. However, male dogs also tended to have faster acceleration time of aortic outflow which was retained after normalisation for heart rate. Mitral Avti appeared to be influenced by gender, with larger Avti in female dogs. Female dogs had significantly lower proportion of early LV filling (Evti/total vti) and higher proportion of late ventricular filling (atrial systole) (Avti/total vti) than male dogs, further supporting this relationship.

Gender was shown to influence mitral inflow parameters in a group of fifty year old human patients (Kangro *et al* 1996), with women having lower E:A velocity ratio than men, which is consistent with this study. Women are reported to have increased mitral A wave and PVF Ar wave, when expressed as a percentage of total forward flow, and also longer durations of A and Ar, than men (Klein *et al* 1998). In this study, no significant association was identified between gender and the various PVF parameters.

B.28.3. *Effect of heart rate*

In this study, the mean R-R interval was calculated from the mean heart rate recorded during the time of image acquisition for the frames which were measured. Few parameters showed a significant relationship to the R-R interval when the 2D or M-

mode echocardiographic results were considered. Forward stepwise and multiple linear regression did identify the mean R-R interval to be an influencing independent variable for EDVI, LAad and LAEI. The 2D LAd:Aod ratio was significantly positively related to the R-R interval. The trend to increasing left atrial area and left ventricular volumes with longer R-R intervals is not surprising, as it indicates enhanced preload associated with longer diastole. Forward stepwise regression did not identify the R-R interval to be a significant predictor of M-mode parameters in most instances. This is in contrast to the studies of Jacobs & Mahjoob (1988a;b). One reason for this may be that the mean R-R interval rather than the preceding R-R interval was recorded. Jacobs and Mahjoob (1988b) showed that the preceding R-R interval did have a significant effect on M-mode echocardiographic parameters in the dog and improved confidence intervals and prediction intervals if they were included in multiple regression analysis with BSA. Jacobs & Mahjoob (1988a;b) also found that there was a non-linear relationship between R-R interval and the M-mode parameter, and transforming the R-R interval by using its square root rendered the data linear. Where there was a dependence on R-R interval shown in this study, the relationship did appear to be linear, but this may merely reflect the relatively low range of heart rates recorded in this population, compared with the large range in heart rate generated by atrial pacing in the Jacobs & Mahjoob (1988a;b) study. MAMmean was also significantly predicted by R-R interval, with gender and age also significant independent variables in a multiple linear regression model.

As expected, many of the Doppler parameters were influenced by heart rate. Aov tended to reduce with longer R-R interval, presumably in association with the expected association between ET and R-R interval, since the vti was not affected by R-R and cardiac output was maintained. An increase in peak velocities with faster heart rates was also reported in two dog breeds by Kirberger and colleagues (1992b). It was logical, also, that the peak and mean acceleration of the aortic flow (dv/dt_{max} and dv/dt_{mean}) tended to reduce with longer R-R interval, in the absence of any significant heart rate prediction of the aortic acceleration time. Vcf followed the same trend as ET under the influence of R-R interval, tending to decline at slower

heart rates. Although stepwise regression did not indicate that the R-R interval was a significant independent predictor for PEP, once the effect of gender had been taken into account, simple linear regression did indicate a weak positive but significant association. This is in contrast with most veterinary studies (Pipers *et al* 1978; Atkins & Snyder 1992). The lack of dependence of the PEP:ET ratio on other variables is consistent with the human and veterinary literature. The fact that the R-R interval is the most significant predictor of the various STIs is supported by the fact that once PEP, ET, the PEP:ET ratio and Vcf were indexed to √R-R interval, all other independent variables were eliminated from the forward stepwise regression model. The exception to this was the acceleration time/√R-R interval, which retained its relationship with gender.

As expected, mitral E duration, E deceleration time, A duration and IVRT were all independently predicted by R-R interval. The E:A velocity ratio increased and Av decreased with lengthening R-R, showing that there is less requirement for active atrial contraction in ventricular filling at slower heart rates, with more time for passive diastolic filling.

Many of the PVF parameters were significantly influenced by heart rate. Arv, Sv and R2v declined at longer R-R intervals, as did the S:D velocity ratio. Longer R-R allow more passive diastolic filling of the LV, which reduces the late diastolic atrial volume and pressure, explaining the trend to reduced mitral A and PVF Ar velocities. With increasing R-R, passive diastolic filling of the left ventricle allows most forward PVF to occur during the D wave, possibly explaining the lower S velocity seen with slower heart rates. The haemodynamic events associated with R2 remain to be confirmed, although Schober and others (1998) suggest that it may represent pressure changes in the left atrium as a consequence of mitral annulus recoil. The S wave is due in part to MAM, and so a reduced R2 in association with reduced S would be logical. The systolic fraction of total forward flow also decreases as R-R interval lengthens. PVF durations increase with longer R-R interval, especially S and

D durations and D deceleration time. The D velocity time integral is also associated with longer R-R interval and the S:D vti ratio therefore decreases.

PAv tended to be lower with longer R-R interval, which reflects similar findings for aortic velocity. Longer R-R intervals were associated with lower tricuspid Av, and increased Ev:Av ratio, mirroring the mitral inflow findings.

B.28.4. *Effect of age*

A major finding in this study was that age significantly negatively influenced some of the 2D and M-mode echocardiographic parameters. Multiple linear regression showed that advancing age was associated with smaller LVIDd and lower LVdv, EDVI, LVsv and ESVI. This is consistent with the M-mode data provided in humans (Gardin *et al* 1979; Henry *et al* 1980), although changes in the left ventricular lumen are not identified in all studies (Gerstenblith *et al* 1977). Modest inverse correlation between age and left ventricular systolic volume is reported in man by Wong and others (1995). Associated with the apparent trend of reducing left ventricular cavity size with age, most marked in diastole, was the finding that the stroke volume and stroke volume index were also affected, tending to reduce in older dogs, despite linear regression showing no significant positive or negative relationship between heart rate (R-R interval) and age; i.e. this trend is not due to faster heart rates in older dogs and it must be presumed that cardiac output is therefore also reduced in the older dogs. Age associated decline in cardiac output is reported in man (Wei 1992). When both parameters were indexed to BSA, the ratio of LVpwd:LVIDd showed a positive correlation with advancing age. The decrease in LVIDd associated with age explains this, although the R value shows a stronger relationship than that expressed between LVIDd and age, implying that wall thickness also increases with age. Although the effect of age on wall thickness had not been a statistically significant trend in this population of Newfoundlands, it is noted to be a common age related finding in man (Gerstenblith *et al* 1977; Gardin *et al* 1979; Henry *et al* 1980; Feigenbaum 1994d). The M-mode parameters showed a non-significant trend that

FS% and EF reduced with age, which is consistent with the age independence of these parameters in man (Gerstenblith *et al* 1977; Gardin *et al* 1979; Henry *et al* 1980). MAMlateral tended to decrease with advancing age, and this relationship became even more pronounced once the parameter was indexed to BSA.

The finding that the M-mode LVIDd decreased with age and that chamber walls were not influenced by age is in contrast to work in Scottish deerhounds. Bodey (1998) reported that age resulted in thinner LV walls and larger LV lumens in Scottish deerhounds. No attempt was made in the Bodey study to distinguish between normal and cardiomyopathic deerhounds and a number of dogs had dilated chambers with relatively thin walls. This finding may indicate that older deerhounds are more likely to develop DCM.

Many human echocardiographic texts publish references for the effect of ageing in children, but information about M-mode and two-dimensional echocardiographic parameters in ageing adults is sparse. However, Feigenbaum (1994d) provides an appendix showing the effect of age from infancy to old age for M-mode parameters, with some graphs of parameter and BSA, with different levels for age groups (20, 40, 60, 80 years). These are based on the work of Henry's group (Henry *et al* 1978; Gardin *et al* 1979; Henry *et al* 1980). These show that the LV diastolic and systolic diameter decreases with advancing age and wall thicknesses increase. The aortic root dimension and left atrial dimension also increase with age, although fractional shortening, ejection fraction and percentage thickening of septum and free walls are not influenced by age (Gardin *et al* 1979; Henry *et al* 1980). Benjamin and others (1992) noted that there were age associated changes in cardiac anatomy with increased left atrial size and left ventricular wall thickness, while reporting on the age-related effects on diastolic function.

The formula of Henry (Henry *et al* 1980) is sometimes used to predict left ventricular end-diastolic diameter in man (e.g. Baig *et al* 1998). It is given as:

$$LVIDd(predicted) = 45.3 \times BSA^{0.3} - (0.03 \times age) - 7.2$$

This formula indicates that there is a positive association with the cube root of body surface area and a negative linear association with age. This was consistent with the data presented from this Newfoundland study, although the relationship to BSA was linear, which may be due to the relatively uniform size of this particular dog breed. Study of the residuals in the linear regression for age, however, showed that the negative relationship was not perfectly linear in Newfoundlands, when the residuals were assessed for normality, although attempts to transform the data did not improve linearity.

B.28.4.1. *Doppler parameters and age*

Increasing age was associated with a decrease in the Aov, Aovti and stepwise and multiple linear regression identified age as a negative predictor of ET. A significant effect of age was evident on many of the mitral inflow parameters. Ev, E:A velocity ratio, Evti and E:A vti ratio, the proportion of early LV filling and the peak filling rate indexed to mitral stroke volume all decreased with advancing age. E duration and deceleration time and E deceleration/√R-R showed a significant positive relationship with advancing age. The changes associated with age identified in Newfoundlands mirror the similar findings in the human population (Manyari *et al* 1985; Bowman *et al* 1988; Nishimura *et al* 1989; Störk *et al* 1990/91; Nishimura & Tajik 1997) and cats (Santilli & Bussadori 1998) (although in cats, there was no age association with E deceleration time). The E wave changes indicate reduced left ventricular compliance with age (Yamamoto *et al* 1996). However, in this study, no age predicted effect on mitral A wave was identified, a finding which is normally reported in the human literature (Störk *et al* 1990/91; Mantero *et al* 1995) and has been reported in a single feline study (Santilli & Bussadori 1998). The exception in this study was that the proportion of late ventricular filling (Avti/total vti) did increase with advancing age. Age is the most significant predictor of trans-mitral

flow patterns in man (Lewis 1996; Mantero *et al* 1998) and this study suggests that this is also true in Newfoundland dogs.

In this study, age was not identified as an independent predictor for IVRT. When IVRT was normalised for heart rate, by dividing by the square root of the R-R interval, there was a trend to increasing values associated with advancing age, but the relationship was weak and not statistically significant.

Age significantly influenced many of the PVF parameters. Increasing age was associated with an increased Arv, Sv, Svti, S:D velocity and vti ratios and increased systolic fraction of total forward flow. These findings are consistent with the human literature (Klein & Tajik 1991; Gentile *et al* 1997) and the findings in cats (Santilli & Bussadori 1998). In cats, no age association was reported for the Ar wave, neither was this a significant association in humans in the study by Gentile and others (1997). The changes affecting the D wave indicate reduced LV compliance (Yamamoto *et al* 1996), which decreases with age, supporting the findings from the mitral E wave data. D wave duration also tended to be shorter with advancing age, despite no significant association being identified for age and R-R interval. The effects of age on the PVF pattern is thought to be due to altered left ventricular relaxation due to ageing (Klein & Tajik 1991).

The mechanisms of the change in diastolic function with advancing age have not been fully explained, although the human literature suggests a few hypotheses, which were reviewed by Wei (1992). Benjamin and others (1992) queried whether other age associated conditions such as myocardial ischaemia, hypertension or diabetes mellitus could be responsible. They proposed that the changes may be due to a combination of cellular hyperplasia, cell death and fibrosis, reduced calcium sequestration and increased passive stiffness (Benjamin *et al* 1992). Although blood pressure was not routinely measured in the Newfoundlands in this study, essential hypertension and myocardial ischaemia resulting from coronary artery disease are uncommon in dogs. It also appears unlikely that diabetes mellitus is prevalent enough

without resulting in other clinical signs in the Newfoundland population to affect these echocardiographic results significantly. However, blood pressure does tend to rise with age in the canine population (Bodey & Michell 1996). It was unfortunate that blood pressure had not been recorded in Newfoundland dogs in this study, although this had been the initial intention, in order to determine systolic wall stress. Because of thick hair coats and fleshy tails, it proved to be time-consuming procedure and usually unrewarding. The lack of significant changes in left ventricular wall thicknesses do not support an increased afterload related aetiology. In the M-mode study of Gardin and others (1979), the age-associated echocardiographic changes were present in a group of 136 adults aged from 20 to 97 years old, in which the mean diastolic and systolic blood pressures varied by less than 15 mmHg between the age groups. In the study by Gerstenblith and others (1977), although systolic blood pressure did increase with age, there was no correlation between systolic blood pressure and wall thicknesses indexed to BSA and hypertensive individuals were excluded from the study. It appears, therefore, that these changes occur in the absence of associated age related increases in blood pressure. Another potential reason for the increasing afterload in the elderly is increased aortic stiffness (Wei 1992). It remains to be proven whether the left ventricular chamber does genuinely decrease in size with age in an individual animal. Serial evaluation of a number of animals over their life-times is required to confirm this.

Wei (1992) reviewed the age related changes affecting the myocardium. Cardiomyocyte volume increases, although numbers decrease. An increased rate of degenerative changes, with lipid, lipofuscin and amyloid deposition and an increase in the amount of interstitial fibrosis and collagen with increased collagen cross-linking all probably increase myocardial stiffness. These changes occur in man even in the absence of coronary artery disease. Diastolic function is further impaired by an age related decline in the rate of relaxation. Relaxation requires more oxygen and energy than the process of cardiomyocyte contraction and Wei (1992) noted that in humans, there was an age dependent decline in arterial partial pressure of oxygen which was quantified as about 4 mmHg per decade. The author proposed that this

mild hypoxia may play a part in the slowed relaxation process. Another factor which may play a role in compromised diastolic function in elderly humans is that an age associated decline in the rate and maximal capacity of calcium sequestration by the sarcoplasmic reticulum and an increase in the net calcium trans-sarcolemmal influx may slow the active relaxation process. It seems reasonable to propose that most of these factors would also apply to non-human species including this Newfoundland population.

PAv tended to decrease with age and tricuspid Ev and E:A velocity ratio also tended to decrease with age, indicating that right ventricular compliance is affected by age in a similar way to left ventricular compliance.

B.28.5. *Assessment of Normal Newfoundlands: comparison with previous studies*

Tidholm and Jönsson (1996) reported that normal Newfoundlands should have a FS% of over 25%, whereas the mean value in this population was only 24.47%. However, 10% of dogs in Tidholm and Jönsson's (1996) control group had FS% <22%, although these authors suggested using FS<22% as a diagnostic criterion for DCM in this breed. In this study, nineteen out of 86 Normal dogs (22%) had a FS<22%, with the minimum FS recorded as 20% (which was an initial diagnostic criterion of normality when categorising Newfoundlands). The reference range for the LVIDd ranged from 3.5 to 6.0 centimetres, and LVIDs from 2.2 - 4.4 centimetres in the control group, which included juvenile dogs (Tidholm & Jönsson 1996), whereas in this study of normal adult Newfoundlands, the maximal recorded LVIDd and LVIDs were 5.49 and 4.12 centimetres respectively, and dogs with LVIDd of 6 cm or LVIDs of 4.4 cm were unequivocally abnormal in the author's opinion. It is not clear whether the dogs in the Tidholm and Jönsson (1996) control group were serially evaluated to confirm normality, although this is also a criticism of this study; not all the dogs in this normal group received a repeat scan. Limited details about normal echocardiographic values for giant breeds, including 27 Newfoundlands as well as great Danes and Irish wolfhounds, were provided by Koch and others (1995).

These workers noted that fractional shortening was lower in giant breeds than published normal values, and suggested that systolic function in giant breeds may be compromised.

For normal cavalier King Charles spaniels, the 2D LAd:Aod ratio was 1.0 ± 0.06 (Häggström *et al* 1994). The equivalent ratio in this study was 1.10 ± 0.11, which is slightly larger, although whether comparison between dog breeds of such diverse size and conformation is valid may be controversial. The M-mode LAs:Aod ratio is normally 0.8 - 1.2 (Bonagura *et al* 1985), which is consistent with the normal Newfoundland group, even though the internal left atrium/left auricular appendage measurement was the method used in this study, rather than a dimension including the posterior aortic wall (Sahn *et al* 1978).

The PEP:ET ratio was longer in this normal Newfoundland group than reference ranges given in the veterinary literature, which range from 0.24 to 0.34 (Pipers *et al* 1978; Atkins & Snyder 1992; Darke *et al* 1993; Amberger & Lombard 1998). It is, of course, still a concern that the ultrasound machine used is still overestimating PEP despite the correction to the data (Appendix B.1.). However, similar STI data is now generated by M-mode or Doppler, and so it appears that this is genuinely a breed specific difference.

These Normal Newfoundlands were compared with the results from normal dogs described by Schober and our group (1998). Systolic function appeared to be lower in the Newfoundland group than the previous study (Schober *et al* 1998) (EF: 41.77% versus 58%, FS: 24.47% versus 31%, PEP:ET ratio: 0.40 versus 0.35, Vcf: 1.38 versus 1.56 circs/s). There were also differences in the transmitral patterns. Ev and Ev:Av were lower (0.61 versus 0.73 m/s and 1.32 versus 1.63 respectively) although Av and Avti were similar between the two studies. Evti and E:A vti ratio were slightly higher in the Newfoundland study. Similar contrasting findings were evident on comparison of the PVF patterns. Sv was similar in both studies, although Dv was much lower (0.37 versus 0.56 m/s), the S:D velocity and vti ratios were higher (1.04

versus 0.7 and 0.81 versus 0.72 respectively) and Arv was slightly higher (0.24 versus 0.2 m/s) in the Newfoundland study. The systolic fraction (Svti/total forward vti) was similar in both studies. These two studies were performed on two different makes of echocardiograph and measurements were made by different observers, which may explain some of the differences. However, age differences between the two groups may also be responsible. The mean age was not given for the Schober study, but most of the dogs were young and the oldest dog was only eight years old. The mean age in the Newfoundland study was 4.35 years old, but older animals were common in the group.

In all but three dogs in this study, mitral A duration exceeded the PVF Ar duration, and the differences were not felt to be significant. This is consistent with the human data, where the majority of normal subjects have longer mitral A duration, although similar "insignificant" outliers are also reported (Abdalla *et al* 1998; Klein *et al* 1998).

The MAM data were consistent with the canine data provided by Schober and colleagues (1997). In both studies, MAMlateral was greater than MAMseptal. The fact that the Schober study included mainly small and medium sized breeds of dogs, and this study included a single giant breed with no significant predictive effect of body surface area or weight, yet both studies generated similar values is surprising. It appears odd that dog size bears no relationship to the magnitude of MAM, but both studies are consistent in this finding. In the normal Newfoundland population, although a weak relationship was demonstrated between FS% and MAMmean and MAMlateral, no relationship was identified between any of the MAM measurements and EF. This relationship is reported in dogs (Schober *et al* 1997) and man (Alam 1991, Pai *et al* 1991, Willenheimer *et al* 1997), although these studies were in patients with heart disease.

B.29. *Comparison between Newfoundland groups*

B.29.1. *2D LV parameters*

DCM, almost by definition, is associated with left ventricular dilatation and it was not therefore surprising to find that the DCM group had larger LVdv than the other groups. Normalisation to BSA also resulted in identification of increased EDVI in the SAS group. Although the LVE group had been defined by M-mode criteria, LVdv showed a non-significant trend to increased volume, although this was associated with a shorter LV systolic length, implying that the LV in the LVE group was more spherical than groups other than the DCM group. The DCM group had a significantly larger LVsv than the other groups (other than the LVE group). The ESVI was very specific, in that the DCM group had significantly greater volume than all other groups.

The low EF associated with DCM is expected. SV was not significantly lower than the Normal or the two dFS groups. This is probably because most dogs in the DCM category had occult disease or were relatively stable on medication. SV was therefore relatively well maintained, utilising the Frank-Starling mechanism. It should also be appreciated that not all the stroke volume is forward, effective flow; the DCM group did have mitral regurgitation of varying severity also.

Both dFS groups had intermediate EF between the Normal and DCM groups. SV was relatively preserved in these two equivocal categories, although tended to be lower in the dF<18% group. The slightly lower EF in the two dFS groups appeared to be mainly due to lower diastolic volume rather than increased systolic volume. It was surprising to find that the LVE group tended to have increased EF, SV and SVI than the other groups. This appeared to be due to a larger diastolic volume with a relatively normal systolic volume. Numbers in the LVE group were small, so identifying statistically significant differences was not always possible. One reason for the difference was that this LVE group did have a trend to a lower heart rate (although not statistically significant). However, normalising for heart rate, by dividing by √R-R, rendered these differences statistically insignificant.

The SAS group showed significantly increased EDVI and slightly higher ESVI than the Normal group, with significantly increased SVI. This may reflect concurrent mitral or aortic regurgitation volume overloading the LV. Certainly, aortic regurgitation was commonly identified in Newfoundlands with even very minor severity of subaortic stenosis, and was rare in Normal dogs (see later).

B.29.2. *2D LA parameters*

As expected, the left atrial diastolic parameters in the DCM group were larger than the other groups, although this was not statistically significant for the LVE group, which showed a trend to larger LAad. The difference was less marked for the left atrial systolic parameters, although the DCM group did show larger LAas and LAls than the dFS<18% group. The dFS<18% group appeared to have smaller left atria than the other groups, based on these RPS four chamber views and the short axis view, which is interesting as this group also showed the smallest LV volumes.

LAas and LAls showed a trend to being larger than normal in the SAS group, particularly once indexed to BSA, although this was not generally statistically significant. This suggests that the left atrium is functioning further up the Frank-Starling curve, due to the increased LA afterload associated with subaortic stenosis and possibly elevated end-diastolic left ventricular pressure or a stiffer left ventricle than normal dogs.

The LAEI was significantly lower in the DCM group, although this trend was also shown in the LVE group. This suggests that there is probably intrinsic left atrial dysfunction as part of the manifestation of DCM in Newfoundland dogs. This lower index in the DCM group may also be explained by the presence of atrial fibrillation in ten out of the 35 dogs in this group, so an absence of atrial systole will certainly have affected this index. However, the LVE group showed a similar reduction in the LAEI and none of these dogs had AF. If the LVE finding is a predictor of dogs

destined to develop DCM, this suggests that intrinsic atrial dysfunction may be an early feature of the condition.

The 2D LAd:Aod ratio supports the suggestion that the DCM and LVE groups have larger left atria than the other groups, although the only significant difference was the smaller ratio identified in the dFS<18% group.

B.29.3. *Mitral regurgitation*

Trivial amounts of MR are common in Newfoundland dogs, with approximately fifty percent of Normal dogs showing CFDE evidence of mitral valve incompetence. The absence of any significant relationship between any of the left atrial parameters and the severity of MR for dogs in the Normal, dFS<18%, dFS18-20% and SAS groups supports a lack of haemodynamic significance. In contrast, dogs with DCM showed a highly significant relationship between MR and LAld, LAls or LAas. This supports the hypothesis that associated with LV and LA dilatation, mitral annulus stretch or dilatation associated disruption of the mitral valve apparatus is responsible for MR. This in turn, by further volume overloading of the LA and LV, results in further dilatation (Keren *et al* 1988b). In DCM, the severity of MR is also determined by the degree of LV dilatation and reduced contractility (Mukharliamov *et al* 1986). However, specific papillary muscle dysfunction may also play a role (Junker *et al* 1993). The mitral regurgitant fraction is reported to be typically about 20% in many human patients with DCM, although it may exceed 40% in some (Keren *et al* 1988b). Junker and colleagues (1993) reported that the presence of MR in human patients with DCM was associated with a higher NYHA class of congestive failure and increased LV volumes and end-diastolic pressure, reduced EF and increased right sided pressures compared with DCM patients without MR. In addition, the one year survival was decreased in the MR group. The limited follow up to date in this study does not allow any conclusions to be drawn about the significance of mitral regurgitation in the DCM group. However, one patient (NF002/P500) had moderately severe MR with only a slowly progressive course.

The LVE group, although not significantly different from the other groups, did have a lower percentage of dogs with no MR, and a trend to more severe MR, which would be consistent with the LV enlargement and associated effects on the mitral annulus. It is probable that the small numbers in this group were responsible for the failure to identify statistically significant differences.

The observation of the presence of MR in a large proportion of normal and abnormal Newfoundland dogs was made without any attempt to differentiate so-called physiological mitral regurgitation from pathological mitral regurgitation, as a consequence of degenerative mitral valve disease or dilatation of the atrioventricular annulus. Wittlich and colleagues (1990) reported the presence of MR in all young healthy adult human individuals evaluated by transoesophageal CFDE. With the less sensitive trans-thoracic approach, Yoshida and colleagues (1988) reported MR in between 38 and 45% of healthy subjects in different age groups, and PW Doppler was positive for MR in 40% of normal subjects reported by Kostucki and others (1986). Trivial or closing regurgitation, as a consequence of valve coaptation, should not be confused with pathology (Wittlich *et al* 1990). Sahn and Maciel (1988) attempted to define criteria for differentiating between innocent and pathological MR. Regurgitant jets which remained very close to the site of valve coaptation and colour M-mode MR duration of less than 100 milliseconds suggest innocent regurgitation. Pathological MR jets accelerate (with colour aliasing and variance) as they pass through a restrictive orifice in a closed valve, and this may be compared with the reversal of the closing volume of blood pushed by the coapting valve.

MR jet area has been used as a method of semi-quantifying the severity of mitral regurgitation in patients (Goldberg *et al* 1988b; Kisslo *et al* 1988). However, factors other than disease severity influence the colour flow mapping of the mitral regurgitant jet, and include pressure difference between the LA and LV, the size and compliance of the LA, the size and configuration of the regurgitant orifice, loading conditions, heart rate and rhythm, orientation of the jet to the ultrasound beam and the gain settings on the ultrasound machine all influence these variables (Kisslo *et al*

1988; Sahn & Maciel 1988; Keren & LeJemtel 1989). Another major factor is the detectability of a colour flow signal. With poor 2D images, colour cannot be encoded into pixels filled with grey scale (Yoshida *et al* 1988), and certainly colour flow imaging was sub-optimal in a large number of Newfoundlands, particularly the larger and older dogs. Despite these limitations, however, a number of authors have mapped jet area by CFDE or PW Doppler echocardiography (Perry 1989; Keren & LeJemtel 1989). The best correlation with angiographic derived severity of MR was the use of the maximal jet area, assessed from three orthogonal planes (right parasternal long axis and short axis views and the left apical four chamber view), and expressing the jet area as a fraction of the left atrial area from the corresponding image (Helmcke *et al* 1987). Day to day variability in mitral regurgitant jet areas of approximately 15% have been reported (Grayburn *et al* 1989). In this study, MR was assessed from the RPS long axis view and the L.Ap. four chamber view, and jet area as an approximate proportion of the left atrium was subjectively assessed (Figure B.5.1.). It is probable that a number of dogs with "trivial" or 1+ MR merely showed normal closure regurgitation.

It was disappointing that CW spectra of MR jets were of sufficiently high quality to allow determination of left ventricular dP/dt in only five individual scans from four dogs. Four of these were in the DCM group and one in the dFS<18% group. An example of the measurement is shown in Figure B.5.2. The mean dP/dt for this limited group of individuals was 2049.40 $\pm$ 1029.57 mmHg/s. With the high prevalence of MR jets evidenced by CFDE, it was hoped that CW spectra of these jets would be obtainable in some normal individuals to allow comparisons between groups of this important, relatively load-independent parameter of contractility. The instantaneous rate of change of left ventricular pressure, peak dP/dt, is reported to show excellent correlation between CW of mitral regurgitant jets and invasive measurements (Bargiggia *et al* 1989; Chen *et al* 1991; Vuille & Weyman 1994). The mean dP/dt_{max} in an experimental dog model with mitral regurgitation, under anaesthesia, was reported as 1266 $\pm$ 701 mmHg/s (Chen *et al* 1991) and Bargiggia and colleagues (1989) gave a range of Doppler derived rate of change in pressure

from the CW MR jet as 410 - 2601 mmHg/second for human patients with a variety of underlying diseases. From these results, the values obtained from these Newfoundland dogs with systolic dysfunction seem rather high. This may be due to the difficulty in measuring very short time durations with accuracy.

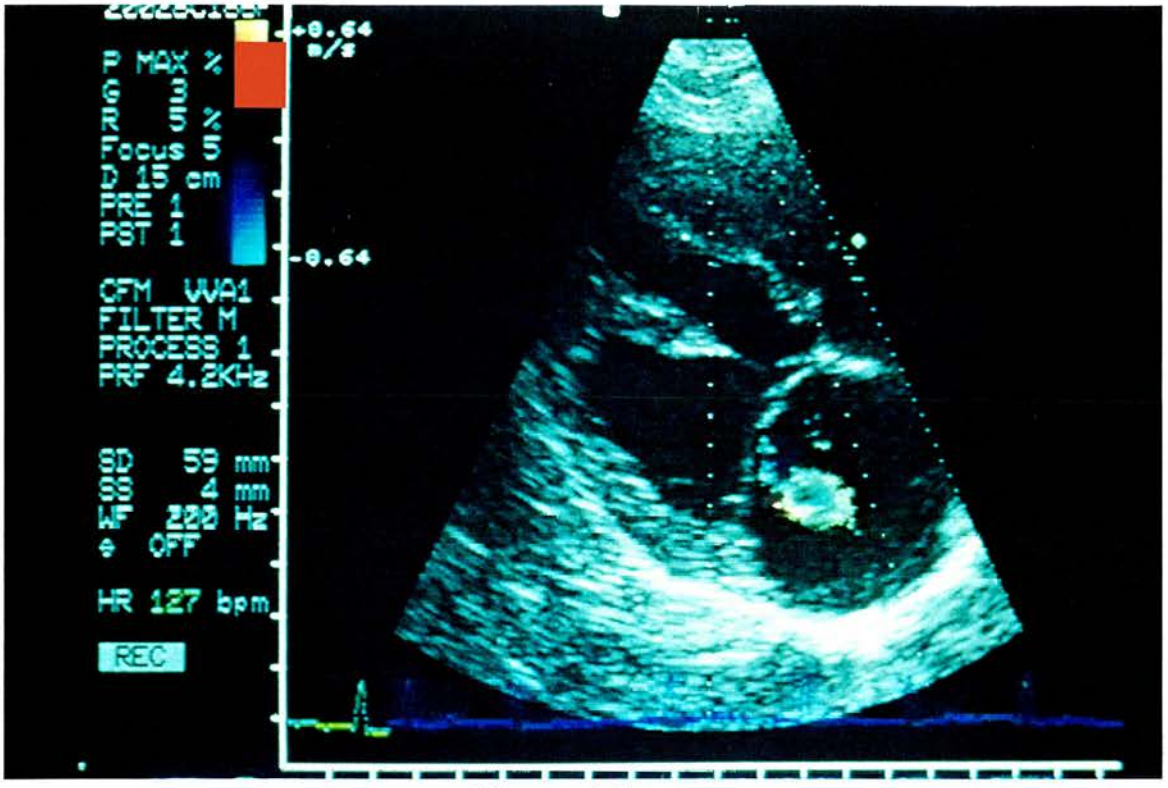


Figure 5.1.

An example of CFDE demonstration of a mitral regurgitant jet within the left atrium of a Newfoundland with DCM and atrial fibrillation (NF002/P500). The jet area proportional to left atrial area from this RPS view was subjectively graded 2+.

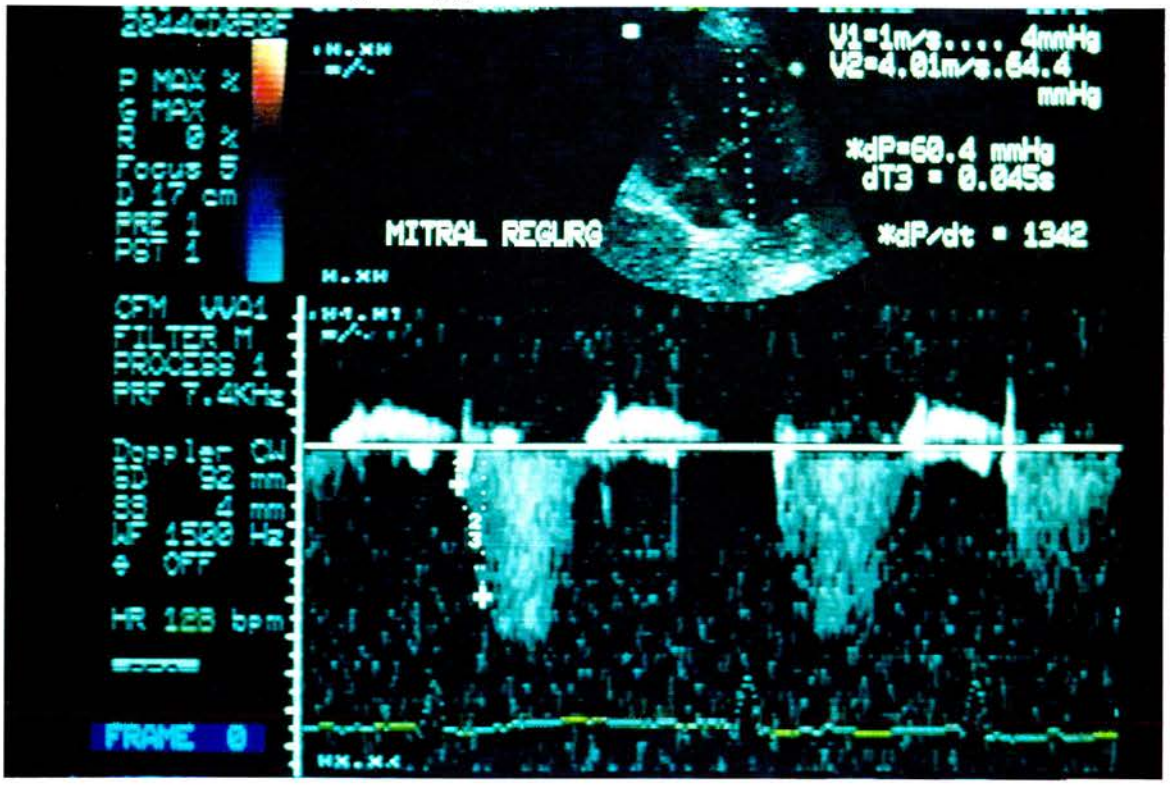


Figure 5.2.

An illustration of the determination of left ventricular dP/dt obtained from a mitral regurgitant jet recorded by continuous wave Doppler from a left apical view. This was from a Newfoundland with DCM and atrial fibrillation (NF002/P500).

B.29.4. *M- mode parameters*

The left ventricular dimensions were predictably and significantly increased in both diastole and systole in the DCM group. LVIDs was rather more specific at differentiating between normal and cardiomyopathic dogs, with little overlap between data points between the Normal and DCM groups. By definition, the LVE group had a large LVIDd, but LVIDs was also significantly greater than the Normal, dFS18-20%, dFS<18% and SAS groups. The DCM and LVE groups' LVIDs was generally larger than the mean + 2sd value generated from the Normal group (40.31 mm), whereas the values for dFS<18%, dFS18-20% and SAS groups were within this value, despite showing a trend to higher mean values than the Normal group. The dogs in both dFS categories tended to have both smaller LVIDd and larger LVIDs than Normal dogs.

IVSd was not significantly different between the groups, although indexing to BSA showed that this measurement was significantly greater in the SAS group, showing some hypertrophy. IVSs, however, was greater in the LVE, SAS and the Normal groups compared with some of the other groups. Little difference was evident between the Normal and the DCM groups for the IVSs measurement, until indexing for BSA. Both dFS categories showed a trend to lower IVSs.

LVpwd showed no significant differences between groups, although indexing to BSA showed thicker walls in the SAS group. LVpws was greater in the LVE, SAS and Normal groups.

There is such a wide spread of data points obtained for %thIVS and %thLVw that it is tempting to dismiss these values as being poorly reproducible. However, statistically significant differences were identified. %thIVS showed a significantly higher value for the LVE group and the Normal group than the dFS<18% group. The DCM group was also low, but not significantly so. There is less data spread for the %thLVpw results, probably since there is not the same difficulty in identifying endocardial surfaces. %thLVpw was significantly low in the DCM group. There

was a non-significant trend to the greatest value for both %thIVS and %thLVpw in the LVE group, which is interesting as other parameters of systolic performance appeared to be non-significantly enhanced in this group.

The LaPlace relationship describes the relationship between chamber pressure (P), wall thickness (w), radius (r) and wall stress, (S) on the left ventricle, if it is considered as a hollow sphere with a certain wall thickness (Levick 1995). The relationship is given by:

$$P = \frac{2Sw}{r} \qquad \text{or re-arranging the formula:} \qquad S = \frac{Pr}{2w}$$

Consequently, the wall stress increases with larger radius or diameter of the LV, and with decline in wall thickness.

The LVpwd:LVIDd ratio may be considered as an indicator of the LaPlace relationship, to indicate the degree of wall stress in the different groups. The LVE group had the lowest value, indicating that the large diastolic diameter of the LV, the measurement which had been selected to define this group, was not associated with proportional LVpw hypertrophy and the wall stress can be inferred to be high in this group. The DCM group was also similarly low, and the geometry of the DCM heart is known to be associated with high wall stress (Levick 1995). The fact that the dFS<18% and dFS18-20% groups had indexes similar to Normal dogs, reflects the smaller LV diastolic diameter identified in these groups, and wall stress may be inferred to be less high than the other groups, although this was not statistically significant. Although the mean value of LVpwd:LVIDd for the SAS group was not significantly different from Normal, this was probably associated with the eccentric left ventricular enlargement with compensatory hypertrophy in this group, there were outlying data points corresponding to dogs with more severe stenosis that did have LVpw concentric hypertrophy, compensating for the high wall stress associated with increased afterload, and therefore a larger index.

The index of sphericity (LVld:LVIDd) was significantly lower in the LVE and DCM groups, showing that the LV enlargement is predominantly along the minor axis, and LV chambers were more spherical. It was surprising that the LVE group had the lowest value, since subjectively, this group was assessed to have relatively normal LV appearance. It is possible that the preserved or enhanced systolic function in this group masked the characteristic appearance of a rounded LV which is subjectively easy to identify in the DCM group.

By definition, the FS% was lower in the DCM, dFS<18% and dFS18-20% groups than the Normal, LVE and SAS groups. Since the Teicholz method of calculating EF uses the same minor axis M-mode dimensions as used for the FS, it is not surprising that identical differences were identified.

Significantly increased mitral EPSS in the DCM group is consistent with the literature. Although there was also a trend to larger LVE EPSS, once EPSS had been normalised to LVIDd, this was no longer evident, and only the DCM group showed a disproportional increase in this parameter. This is due to both LV dilatation and decreased mitral stroke volume as a consequence of the systolic dysfunction so diminishing mitral leaflet opening.

Increase in M-mode LAas is expected in DCM, particularly once the dogs become symptomatic, so this finding was not surprising. There was also a trend to higher LAs in the LVE group. Subjectively, dogs in this group had relatively normal appearance to the LA and LV, which suggests that the LA enlargement is in proportion with the LV enlargement. As expected, there was no significant difference in Aod between the various Newfoundland groups, once this measurement had been normalised to BSA. It was surprising that the M-mode LAs:Aod ratio was not significantly increased in the DCM group. However, M-mode measurements are difficult to obtain through consistent regions of the left atrium or left auricular appendage once the aorta is bisected by the M-mode cursor and the altered LV geometry of the DCM heart makes this more difficult still. Additionally, many dogs in the DCM group were

asymptomatic and so are presumed not to have significant elevated LA pressure, and therefore the left atrial enlargement is less pronounced than in symptomatic DCM cases. It was surprising to identify a trend for an increased ratio in the LVE group, particularly as cardiac morphology had been subjectively assessed as being relatively normal. The anterior aortic root excursions associated with systole were a good subjective indication of systolic function (Figures B.6.1. & B.6.2.), although no attempt was made to quantify this. Some DCM Newfoundlands also demonstrated early systolic partial closure of the aortic valve on M-mode (Figure B.6.2.). This is a common M-mode finding in human DCM patients (Gardin *et al* 1984).

MAM (septal, lateral and mean) was reduced in the DCM group (Figures B7.1. & B.7.2), which is consistent with the findings reported by Schober and others (1997) in dogs and Alam and colleagues (1990) in humans with DCM.

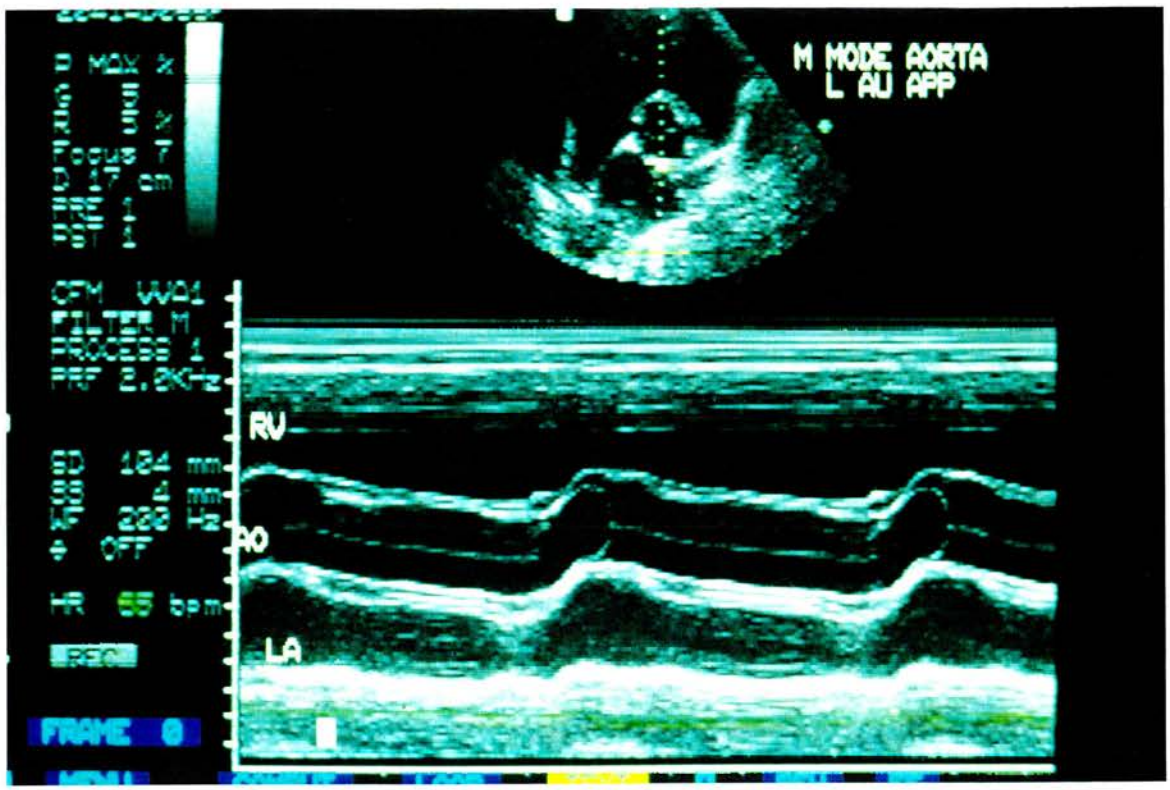


Figure 6.1.

Example of good anterior aortic root systolic excursions subjectively interpreted as representing good systolic function, from an aortic M-mode from a Normal Newfoundland.

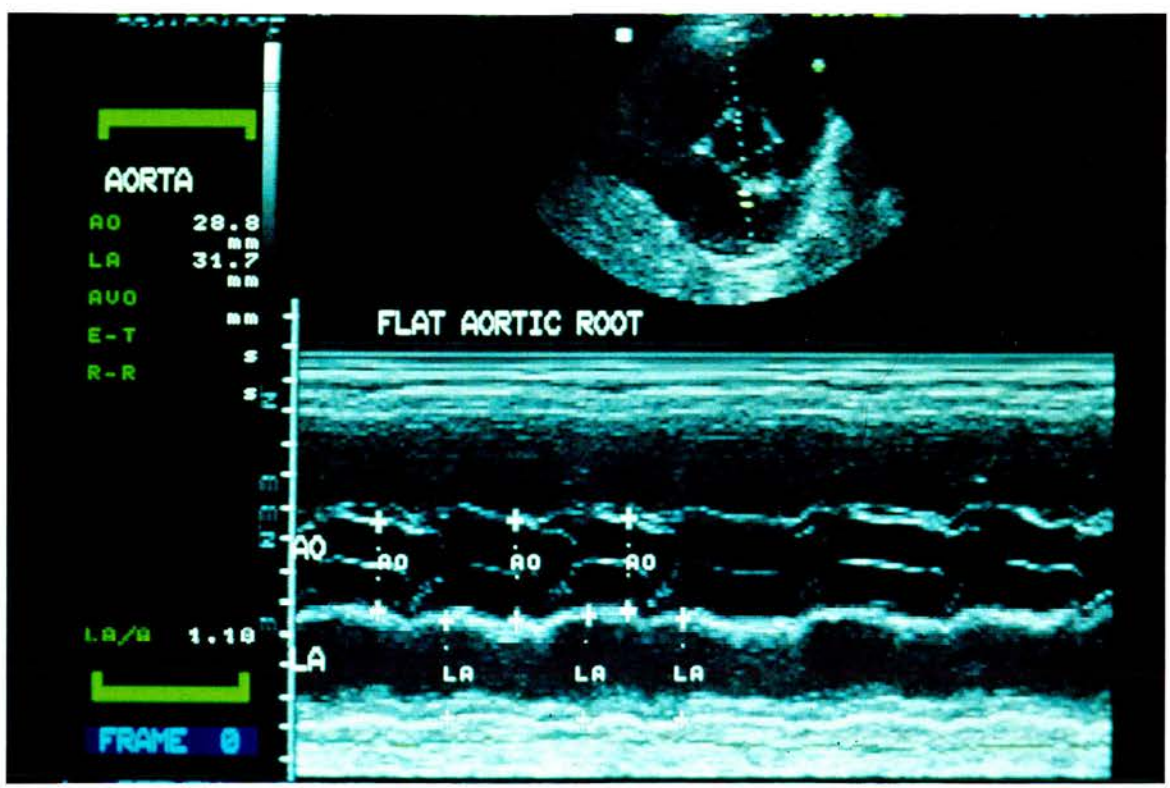


Figure 6.2.

Aortic M-mode showing a "flat" aortic root with minimal systolic anterior excursion, consistent with poor systolic function from a Newfoundland with dilated cardiomyopathy and atrial fibrillation. Early systolic partial closure ("notching") of the aortic valve is also demonstrated.

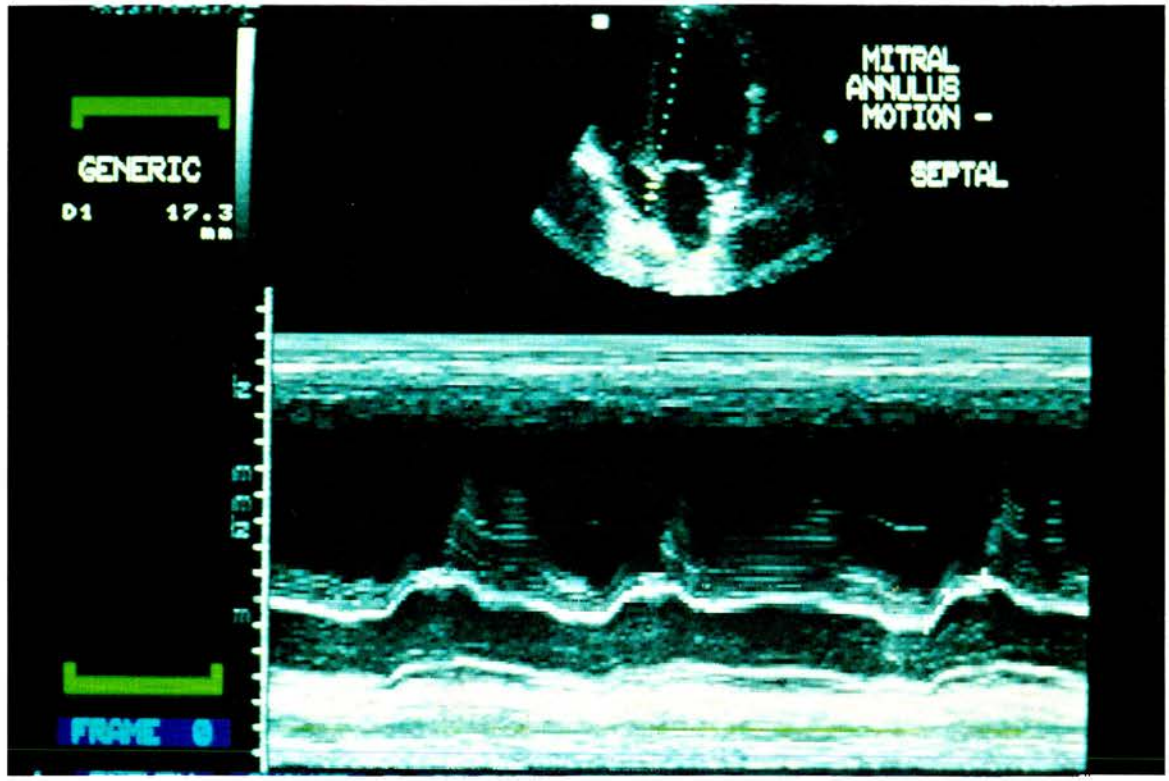


Figure 7.1.

Example of good mitral annulus motion, from M-mode of the septal annulus from a four chamber apical view. This is consistent with good systolic function, from a Normal Newfoundland.

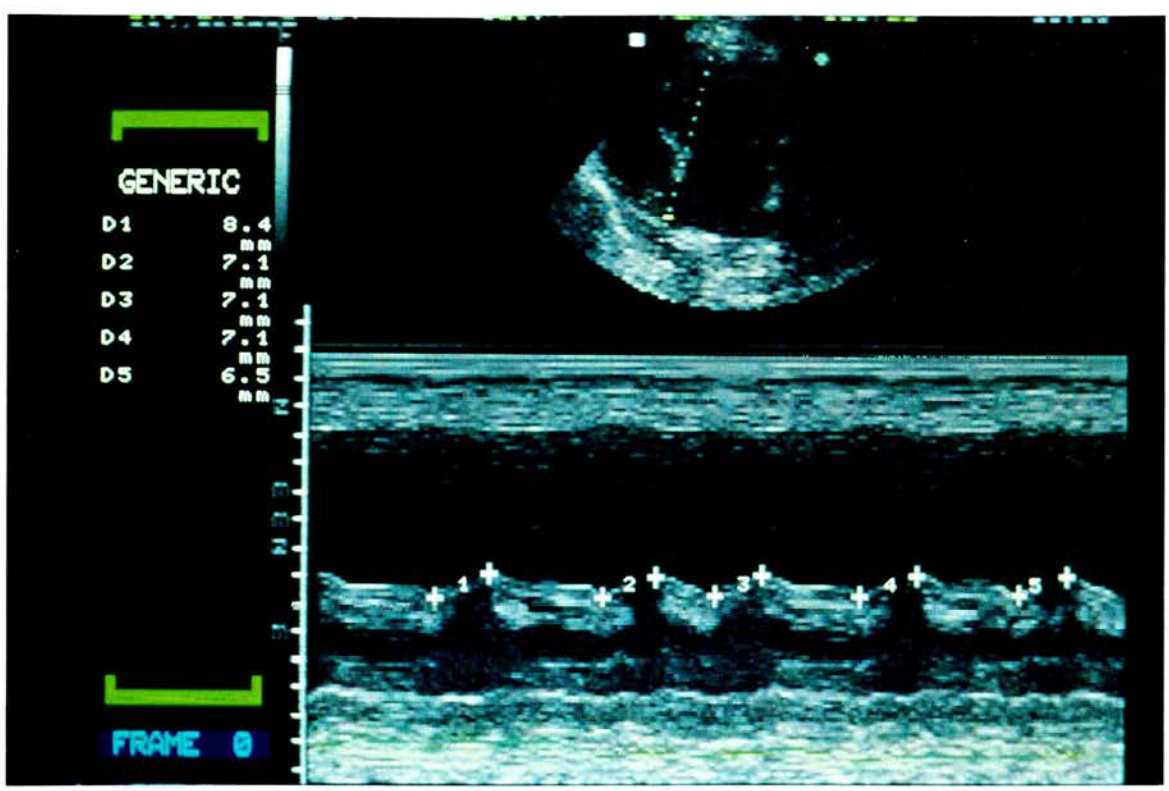


Figure 7.2.

Mitral annulus motion from a Newfoundland with DCM and atrial fibrillation. This is an M-mode of the septal annulus from an apical four chamber view. It shows reduced MAM, consistent with poor systolic function.

B.29.5. *Aortic flow parameters*

There was a trend to lower Aov in the DCM Newfoundlands than the other groups, with velocities from the dFS<18%, dFS18-20% and LVE groups being intermediate between the DCM and Normal Newfoundlands. There was, however, significant overlap between all groups and this, as well as inclusion of the SAS group, with high aortic velocities by definition, probably explains the lack of statistical significance in these trends. Similar trends were also shown in the aortic vti, although the Normal dogs did have significantly higher aortic vti than dFS<18% and DCM Newfoundlands. The trend to lower Aov and Aovti in the DCM group is consistent with the findings in man by Gardin and colleagues (1983).

Since peak and mean acceleration are parameters of systolic function, it was not surprising that dogs with other evidence of impaired systolic function, in the DCM, dFS<18% and dFS18-20% groups, showed lower dv/dt_{max} and dv/dt_{mean} than the Normal dogs. There was no significant difference between the Normal and LVE groups. This offers further evidence that the LVE group, if indeed it is a predictor of dogs destined to develop DCM, have many aspects of systolic function well compensated, presumably by utilisation of the Frank-Starling mechanism. Dogs with SAS had significantly higher dv/dt_{max} and dv/dt_{mean} than most of the other groups, which is expected as they require to have enhanced systolic function to compensate for the increased afterload due to a fixed obstruction.

DCM is known to be associated with increased PEP and slightly lower ET, with an increased PEP:ET ratio (Darke 1994; Amberger & Lombard 1998) and this study supports these findings. There is little overlap between the data for PEP and PEP/√R-R interval between the Normal and DCM Newfoundlands, and dogs in the dFS<18%, dFS18-20% and LVE groups had intermediate data ranges, with surprisingly little overlap between these groups and the Normal Newfoundlands. Differences between the Newfoundland groups were less evident with respect to the ET or ET/√R-R, although values tended to be lower in the DCM group. The PEP:ET ratio is significantly longer in the DCM group than the Normal Newfoundlands, with

little overlap between the groups. The PEP:ET ratios for the dFS<18%, dFS18-20% and LVE lie between the DCM and Normal groups, with gradual decrease between the groups. STIs appear to be rather sensitive and specific at distinguishing Normal from DCM Newfoundlands. This is in marked contrast to the reports by Calvert and Brown (1986) and Darke and colleagues (1993), who did not find any significant difference between cardiomyopathic and normal dogs.

There was no significant difference between Normal and SAS groups for PEP or PEP/√R-R, although the values for the SAS group tended to be slightly lower. There is no statistically significant difference between the SAS and Normal groups for the PEP:ET ratio. This is surprising, as left ventricular hypertrophy in the absence of systolic dysfunction is normally associated with an increased PEP and PEP:ET ratio. This is thought to be due to elevated end-diastolic LV pressure and possibly to decreased preload (Matsuno *et al* 1988). However, SAS was not severe in this group, and significant hypertrophy was not evident.

The aortic flow acceleration time or accel/√R-R was significantly longer in the DCM group than Normal or SAS Newfoundlands. There was some overlap between Normal and DCM groups apparent, which is consistent with the findings of Gardin and others (1983). Dogs in the dFS<18%, dFS18-20% and LVE categories showed similar medians, which were intermediate between the DCM and Normal groups. The acceleration time tended to be shorter in the SAS group, especially after indexing to √R-R. As acceleration time is another indicator of LV systolic function, these findings are to be expected.

The Vcf data for the Newfoundland groups follows the expected trends of FS%. There is little overlap between the Normal and DCM Newfoundlands, with both dFS groups reflecting the DCM group, with little overlap of Normal data. The Vcf for the LVE group, however, is very similar to normal data or SAS data. There appears to be little advantage of using Vcf over the use of FS%.

B.29.6. *Sensitivity and Specificity and Predictive value of PEP or the PEP:ET ratio in distinguishing between Normal and DCM Newfoundlands*

There was surprisingly little difference in whether PEP alone or the PEP:ET ratio was used to differentiate between the Normal and DCM Newfoundlands, although the sensitivity results for the PEP:ET were slightly greater. One limitation of this study was that the final status of the Newfoundlands in each of the groups had not been confirmed in most cases. Indeed, a large number of dogs had not been rescanned. A major assumption that the inclusion of a scan in a particular category represents the conclusive status of a dog must be a major criticism of this study.

From scrutiny of the data, the use of a cut-off of two standard deviations above the mean is too conservative, although it does result in very high specificity for both PEP and PEP:ET ratio (98.8% in both cases) and high positive predictive value (95.5% in both cases). Sensitivity was improved by using a cut-off of one standard deviation above the mean, but at the expense of specificity and positive predictive value. If the option was made for a criterion for the diagnosis of systolic dysfunction as a PEP:ET ratio >0.460, the sensitivity was 85.3%, specificity 82.9%, positive predictive value 67.4% and negative predictive value of 93.2%. This criterion was positive for 51.7% of the dFS<18% group, 45.8% of the dFS18-20% group and 25% of the LVE group.

The one dog in the SAS group with increased PEP:ET ratio (NF023/P248 Scan NF7/7) also had an increased LVsv, LVdv and M-mode parameters, suggesting that this dog was developing DCM, by the time of his second scan (SAS group; scan NF13/8). However, his STIs had become normal. This dog certainly has a positive family history for DCM and it is interesting to speculate whether the PEP:ET ratio was predictive of the development of a myocardial insult, or whether it reflects a transient insult. Only further evaluation of this individual will confirm the progression.

The significance of STIs in the identification of dogs with early DCM still requires to be confirmed with serial evaluation of these dogs with abnormalities in the future. It is likely that the cut-off criterion of one standard deviation above the normal mean is still too conservative and this requires to be refined in the future. However, this study shows that STIs, in particular PEP:ET, are sensitive indicators of systolic dysfunction, which are unaffected by regional wall motion abnormalities and they do not require any geometrical assumptions to be made. Obviously, the value of the PEP:ET criterion in the diagnosis of DCM is enhanced if other classical and more conventional features of the disease are also present.

Although an alternative criterion could be PEP >0.082 seconds, the dependency of this parameter of heart rate in normal Newfoundlands makes it less suitable as a diagnostic criterion, particularly in individuals with slow heart rates. Similar results were evident to the PEP:ET criterion in assessing the other Newfoundland groups, although 5/8 dogs in the LVE group (62.5%) had PEP $\geq$ 0.082 seconds. However, it has already been noted that dogs in the LVE group tended to have lower heart rates than dogs in the other Newfoundland categories.

B.29.7. *Aortic insufficiency*

Aortic insufficiency was not common in Newfoundlands without any aortic valve or left ventricular outflow tract pathology. It was absent in between 70.8% and 89.7% of dogs in the Normal, DCM, dFS<18%, dFS18-20% and LVE groups. In contrast, aortic insufficiency was present to some degree in 67.5% dogs with aortic velocities in excess of 1.7 m/s, a difference which was highly significant (χ^2 analysis: $p<0.001$).

In general, no significant relationship was identified between peak aortic velocity and the grade of aortic insufficiency, although the dFS<18% group actually did show a modest relationship which just achieved statistical significance ($R=0.433$; $p<0.05$). However, the SAS group showed a more significant relationship between these two parameters ($R=0.422$, $p<0.01$) with more severe aortic insufficiency with higher aortic velocities in the aortic stenosis group. It is logical that the presence of

congenital abnormality of the aortic valve or subvalvular region may result in valvular insufficiency as well as stenosis. However, it should be noted that the grade of aortic regurgitation was never more than mild, and it is unlikely that the aortic insufficiency identified in any of the groups was haemodynamically significant. In the SAS group, which tended to have larger left ventricular chamber dimensions from 2D and M-mode echocardiographic methods, the degree of aortic insufficiency was not judged to be sufficient to result in LV volume overload, although this was an entirely subjective opinion.

The relationship between subcostal aortic velocity from all Newfoundland groups and the CFDE record of aortic insufficiency identified by linear regression analysis ($R=0.445$, $p<0.001$) is difficult to explain in the groups where aortic valve or subvalvular pathology was not identified.

Aortic regurgitation has been variously reported in normal humans as never occurring (Yoshida *et al* 1988), to occur in 26% of patients (Kostucki *et al* 1986) or to occur in 68% of normal individuals, with very short duration (Wittlich *et al* 1990). The discordant results probably merely reflect the sensitivity of the detection system; the latter study used transoesophageal CFDE. It appears to be an uncommon finding in Normal Newfoundlands assessed by trans-thoracic echocardiography. Although the jet area and length was used to subjectively assess the severity of any AoR identified in this study, this was probably inaccurate. Switzer and colleagues (1987), in an *in vitro* model, and Perry and co-workers (1987), in patients with CFDE, showed that the jet width at the origin, relative to the short axis area of the left ventricular outflow tract at the same level, was the best predictor of the severity of aortic regurgitation.

B.29.8. *Mitral inflow parameters*

Ev was significantly higher in the SAS and DCM groups. In the DCM group, there also a trend to lower Av. The apparent differences between groups for Ev and Av were no longer apparent after normalising for total vti. There was no significant difference between groups for the E:A velocity ratio, although it was higher in the DCM and LVE groups than the other groups. As the peak mean E:A velocity ratio was 1.48 (LVE group), no group showed evidence of restrictive physiology or abnormal relaxation based on this parameter. It was interesting to note that the SAS group had higher Ev and Av than the other groups, although the E:A ratio was not altered. The SAS group also had the highest total vti than the other groups, although this was only significantly different from the dFS<18% group.

Although there was no significant differences between groups for Evti, some significant differences between groups were apparent for Avti. The DCM and dFS<18% groups showed a trend to lower Avti than the other groups. However, there were no statistically significant differences between groups for the E:A vti ratio, although it was greatest in the DCM group. No statistically significant difference could be confirmed between groups for the proportion of early LV filling (Evti/total vti) or late LV filling (Avti/total vti). Although no attempt was made to exclude dogs with atrial fibrillation from the data (A wave data was treated as "missing"), a trend was identified for a higher proportion of early filling and lower proportion of filling subsequent to atrial systole in the DCM group. Since there was no other confirmation of restrictive physiology or abnormal mitral filling pattern in the DCM, this result may reflect the intrinsic atrial dysfunction identified in this group, determined by the LAEI. However, a similar trend was identified by Darke and others (1993), with AF excluded from their DCM study group.

Although there were apparent differences for E duration and E deceleration time between the Newfoundland groups, these appeared to be related to heart rate as no significant differences between groups was evident after normalising to $\sqrt{}$R-R interval. From these data, since no group exhibited unequivocally restrictive

physiology, it was not possible to define a mitral E wave deceleration value as being consistent with a restrictive pattern, such as is commonly used in human echocardiography (Little & Cheng 1998; Hurrell *et al* 1998), where values <130 milliseconds are associated with a grave prognosis and values more than 150 milliseconds are regarded as being relatively normal. In the Normal Newfoundland population, E deceleration time was 117 milliseconds. It is apparent that definitions of restrictive physiology cannot be merely extracted from the human literature, and confirmed cases of restrictive physiology are required in dogs (perhaps with severe, end-stage DCM), to attempt to define these values further, probably indexed to √R-R interval.

No statistically significant differences were identified between the groups for IVRT. However, after correcting for heart rate, (IVRT/√R-R), it was apparent that this result was much greater in the DCM group (0.095), compared with all other groups (Normal, 0.080; dFS<18%, 0.081; dFS18-20%, 0.084; LVE, 0.085 and SAS, 0.080). This result was surprising when so many of the DCM group had mitral regurgitation to some degree, which implies that an isovolumic relaxation phase cannot occur. This may be because MR was not severe in any dog. However, Keren and others (1986) found that the majority of MR occurs during the ejection phase (79%) rather than the pre-ejection period (13%) in human patients with DCM, in contrast with the angiographic reports that 50% of regurgitant volume occurs during the pre-ejection period in patients with valvular heart disease. This finding explains why a distinct IVRT phase occurs in DCM, despite the presence of significant mitral regurgitation (Keren *et al* 1988b). This trend, even though not statistically significant, to prolonged IVRT in the DCM group is consistent with impaired relaxation.

It was disappointing that so few statistically significant differences between Newfoundland groups could be identified. It had been hypothesised prior to this study that diastolic dysfunction may precede systolic abnormalities or changes in chamber dimensions, although this is apparently not true. It may be that the presence of MR may have played a part in masking abnormalities of diastolic function,

particularly as the DCM group were stable and most individuals probably did not have elevated LV end-diastolic pressure. The confounding influence MR in the use of trans-mitral filling patterns in DCM has been noted by a number of authors (Takenaka *et al* 1986; David *et al* 1989; Lavine & Arends 1989).

B.29.9. *Pulmonary venous flow*

Arv was found to be significantly increased in the SAS group. It was striking that a significant number of data points exceeded the 90% percentile in the Normal group. This had been observed during the scans; a number of dogs showed high Arv without having any other clinical or echocardiographic evidence of heart disease. Some of these dogs were stressed during the examination, judged by observation and from questioning the owner, although they were not tachycardic. Such a dog, confirmed to be Normal, is shown in Figure B.8.1. (NF063/P244 scan 11/1). Her repeat scan (scan 17/5) no longer showed this abnormality. It is difficult to explain this finding physiologically.

No significant differences were identified between groups for the Sv although differences were evident for the Dv, with a trend to higher velocities in the DCM group. Although there was no statistically significant difference in the Sv:Dv ratio between groups, it should be noted than values are similar to or greater than 1.0, even in Normal Newfoundlands (1.04). Although an age related increase in the Sv:Dv ratio is reported (Klein & Tajik 1991; Gentile *et al* 1997) and was identified in the Normal Newfoundlands in this study, the ratios in all groups are much higher than those reported in normal dogs by Schober and colleagues (1998). There were similar findings for the S:D vti ratio in Normal dogs in this study. However, the S:D vti ratio in the DCM and dFS<18% and dFS18-20% groups were similar to the normal value of 0.72 (Schober *et al* 1998).

R2v showed a trend to higher values in the SAS and DCM groups. If this parameter does indeed reflect mitral annulus recoil (Schober *et al* 1998), it is surprising to find relatively large (although not statistically significant) values in the DCM group,

which tended to have lower MAM and probable recoil. As the R2 wave, with the other components of pulmonary venous flow, reflect pressure changes within the left atrium, the relatively increased R2 wave velocity in the DCM and SAS groups may merely reflect the fact that the left atrium is less compliant than in Normal dogs.

The DCM group tended to have a lower Svti, S:D vti ratio, total vti and systolic fraction of total forward flow than the other groups, although most of these parameters failed to achieve statistical significance. Durations of the various wave components of the PVF, even when indexed to heart rate, do not appear to be informative at distinguishing between Newfoundland groups.

Some dogs with mitral regurgitation showed reverse systolic flow as a consequence of mitral regurgitation (Figure B.8.2).

Again, it was disappointing that so few significant differences between Newfoundland groups were identified. Although there were trends shown in the DCM group compared with Normal dogs, few of these achieved statistical significance. This may, in part, be due to the fact that the majority of the DCM group had mild disease. Many were not in congestive failure, or failure was well controlled by medication. Certainly, it is apparent that PVF evidence of diastolic dysfunction does not appear to be a predictor of dogs destined to develop DCM. Typically, in human patients with DCM, peak S wave velocity and the S:D velocity ratio are significantly lower than normal individuals (Kranidis *et al* 1994).

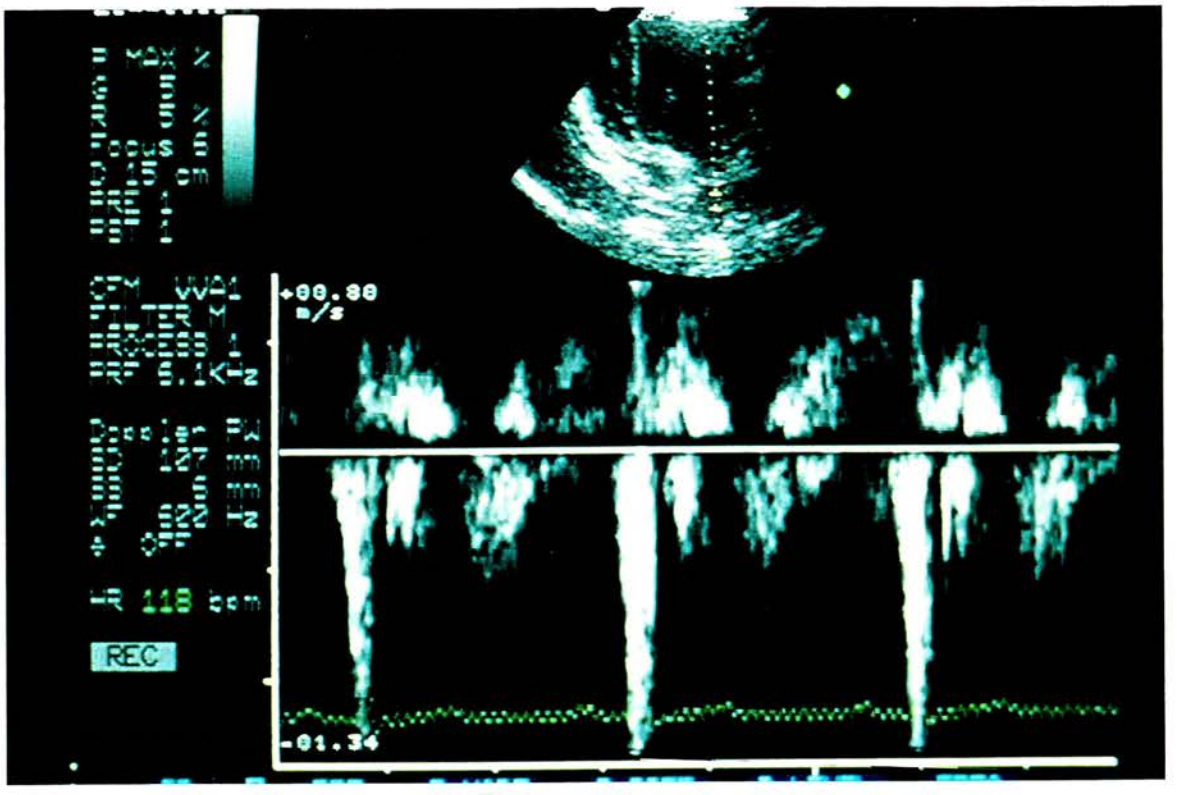


Figure 8.1.

Pulmonary venous flow pattern from a Newfoundland with an otherwise normal scan (NF063/P244), showing very large atrial reversal (Ar) waves with velocity of >1.0 m/s. The cause of these is unknown, but it was speculated that they may be associated with stress.

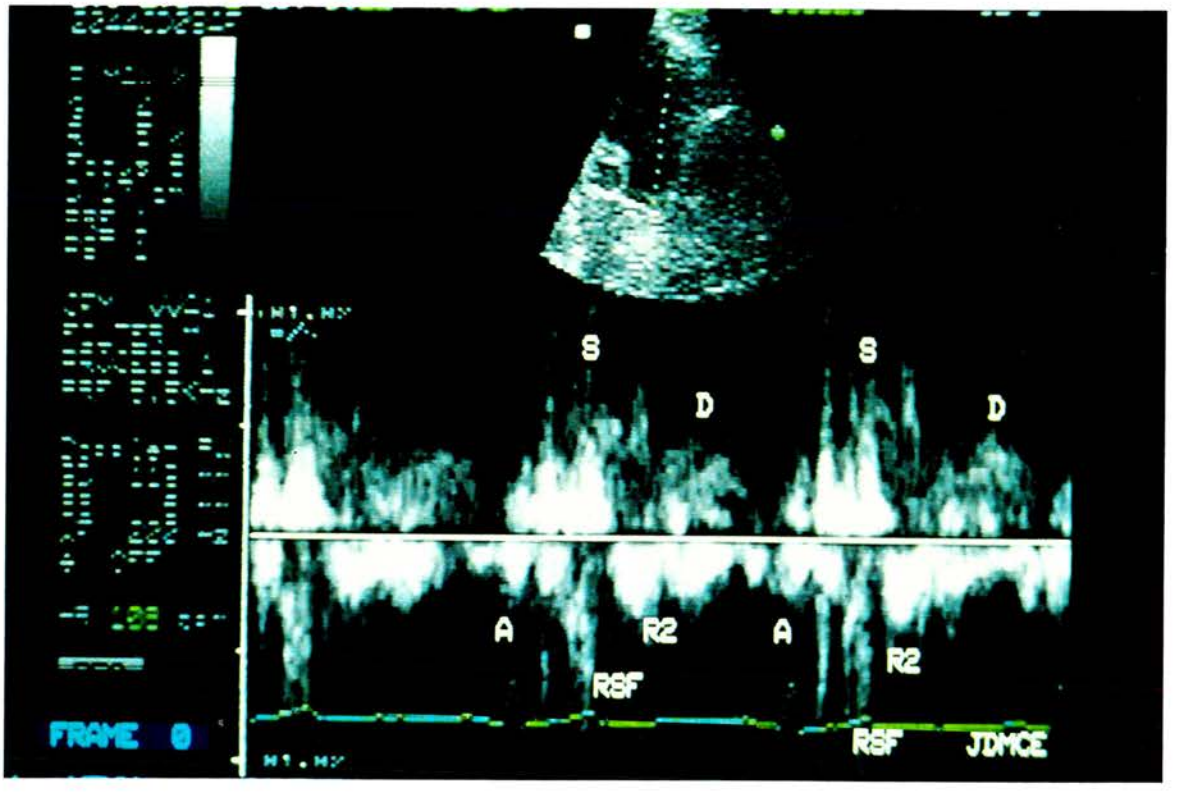


Figure 8.2.

Pulmonary venous flow pattern from a Newfoundland with occult DCM showing reversed systolic flow (RSF) as a consequence of mitral regurgitation. The other components of PVF are labelled. PVF S wave > D wave.

B.29.10. *Pulmonary artery parameters*

PAv from RPS or LPS views were significantly higher in the SAS group, although in no case was a velocity consistent with a diagnosis of pulmonic stenosis identified. The pulmonic velocities were higher from Normal Newfoundlands than in the DCM, dFS<18%, dFS18-20% and LVE groups, from both windows, although these differences were only statistically significant between Normal and the DCM and dFS<18% groups. The trend to lower pulmonic velocities in the DCM group is in contrast to the findings of Gardin and colleagues (1983), who noted no difference in peak pulmonic velocity between normal and DCM human patients.

Increased pulmonic velocities have been reported in association with aortic stenosis in boxers (personal communication, A.French), and this prompted the investigation of a relationship between pulmonic artery velocity and subcostal CW peak aortic velocity. Although a significant relationship was identified, there was also a moderate but highly significant relationship between RPS and LPS PAv and subcostal CW Aov when data from all Newfoundland groups were combined (R values 0.576 (RPS) & 0.546 (LPS) and $p<0.001$). It is therefore probable that the increased pulmonic velocities in association with SAS do not represent a minor degree of contralateral semilunar valve stenosis, but merely represent the fact that the cardiac outputs of both ventricles must be equivalent. Since right sided function was not investigated in more detail in this study, further conclusions could not be drawn.

Pulmonic insufficiency was identified by CFDE in between 27.6% and 50% of Newfoundlands in this study. This was never more than mild and was subjectively assessed as being haemodynamically insignificant in all groups. There was significantly more pulmonic insufficiency in the SAS group and the dFS18-20% groups, although the significance of the relationship in the dFS18-20% group is difficult to explain.

Pulmonic regurgitation has been reported in 70% of normal dogs (Yuill & O'Grady 1989), and is common in normal humans. It was reported in between 28 - 88% of subjects reported by Yoshida and others (1988), with an apparent age related decline in frequency, and 92% of subjects studied by Kostucki and others (1986).

B.29.11. *Tricuspid valve parameters*

The SAS group tended to have higher tricuspid Ev and Av than other groups, although this was not statistically significant for all groups. The DCM group tended to have a lower tricuspid Av, but no significant differences were identified between the groups for the tricuspid Ev:Av. Few conclusions can be drawn from the limited analysis of right sided function in this study.

Tricuspid regurgitation was recorded in between 0% and 36.1% of Newfoundlands in the various groups. TR was identified in a significantly higher proportion of DCM dogs than dogs in other groups (χ^2 analysis: $p<0.05$). This finding is consistent with the fact that some right ventricular involvement will also be evident in DCM, with possibly stretching of the tricuspid annulus with the consequence of tricuspid incompetence. A number of Newfoundlands in the DCM category were noted to have biatrial enlargement.

In normal human subjects, TR has been variously reported to occur. Yoshida and colleagues (1988) reported that 15 - 77% of people in different age groups had TR, with an apparent age related decline. TR was identified in 44% of patients by Kostucki and others (1986) or 100% of patients in the trans-oesophageal echo study by Wittlich and colleagues (1990). It is also reported in 50% of normal dogs (Yuill & O'Grady 1989).

B.30. *Dilated cardiomyopathy category*

Thirty five scans were obtained from 29 individual dogs. In this group, sixteen dogs had only one scan. Eight dogs in this group had had a previous scan in another category before the subsequent scan fulfilled the criteria for the diagnosis of DCM. Four dogs in this group had repeat scans, both in this DCM category and two of these had received a third scan. Of the 35 scans, ten dogs were reported to be symptomatic by the owners at the time of the scan (overt DCM) and were either on therapy or therapy was advised after the scan. No attempt was made to monitor therapy or investigate the relationships of any of the echocardiographic parameters with therapy. Twenty five scans were from dogs not reported to be symptomatic (occult DCM).

Atrial fibrillation (AF) was identified in seven dogs with overt DCM and three dogs with occult DCM. Ventricular premature complexes (VPCs) were identified in two dogs with overt DCM (one in association with AF) and seven dogs with occult DCM, one of whom had a paroxysm of monomorphic ventricular tachycardia during echocardiographic evaluation (NF070/P274, scan NF18/12). Two scans from the same dog with occult DCM (NF042/P542 scans NF9/5 & NF13/5) showed frequent supraventricular premature complexes. Only two scans with overt DCM and twelve scans from dogs with occult DCM did not have any arrhythmia identified. One dog with occult DCM appeared to have first degree atrioventricular block, although this was measured from the ECG on the echocardiographic recording. The presence of arrhythmias in association with echocardiographic abnormalities were felt to support the decision to include dogs in this DCM category rather than other categories. It is apparent that arrhythmias, particularly atrial fibrillation, became more prevalent during progression of the disease course.

Systolic heart murmurs were reported in seven of the ten scan records from dogs with overt DCM, and only six of the scan records from dogs with occult DCM. The murmurs in all cases were consistent with mitral regurgitation.

The echocardiographic / Doppler parameters in this study were consistent with those expected in DCM, and those reported in human DCM (reviewed by Levine, 1994). The DCM group had larger LV volumes and M-mode dimensions than other groups, especially in systole. The LV ESVI and M-mode LVIDs appear to be more specific and sensitive at distinguishing between DCM dogs and other Newfoundland groups. The DCM group showed a trend to thinner walls, especially in systole, and a lower percentage thickening of the IVS and LVpwd. EF and FS were reduced and EPSS was increased. The various 2D or M-mode LA parameters all tended to be increased relative to the other Newfoundland groups, and left atrial dysfunction was identified in DCM dogs by the LAEI, even in dogs in sinus rhythm. Twenty seven scans (77.1%) showed MR of variable severity. The fact that MR grade correlated with LAls and LAld as well as the LAas supports the fact that MR is a consequence of dilatation of the mitral annulus.

During the course of this study, it was evident that dogs with a firm diagnosis of DCM showed increased sphericity of the chamber. In an attempt to quantify this subjective finding, the ratio of diastolic LV length: LV minor axis dimension (LVld:LVIDd) was used and was found to be significantly reduced in the DCM group.

In dogs with overt DCM, there appeared to be two forms to the disease. While some dogs developed marked LV dilatation and hypokinesis, with LA enlargement appearing later in the course as clinical signs became evident, other dogs showed only minor LV dilatation, although increased sphericity was evident, but marked, disproportionate left or biatrial enlargement (usually in association with atrial fibrillation) (Figures B.9. - B.11.). Dogs in the former group appeared to deteriorate more rapidly. The group without marked LV dilatation were usually older with slower progression, or even echocardiographic improvement with appropriate treatment. They may have significant MR (Figure B.5.1. & B.5.2.), although LA enlargement was subjectively judged to be disproportionate to volume over-loading. LA enlargement may result in dilatation of the mitral annulus and MR (Tanimoto &

Pai 1996), even in the absence of LV dilatation. However, some mitral valve degeneration was evident on gross and histopathology in the old Newfoundlands (personal communication, Dr. R. Else). It is possible that the age of onset does determine the course of the disease. It has already been noted that the left ventricular cavity tends to be smaller in older, normal dogs, and it was postulated above that this may be due to increased collagen deposition and collagen cross bridge formation. This may have a protective effect in dogs with a later age of onset of DCM, preventing massive left ventricular dilatation. It is also possible that this represents the cardiomyopathy described as "mildly dilated congestive cardiomyopathy" (MDCM) described in man (Keren *et al* 1988a; D'Cruz *et al* 1992), which also is associated with a more favourable prognosis. Keren and colleagues (1990) gave a detailed clinical and pathological description of MDCM, which is characterised by minimal myofibrillar loss.

The aortic Doppler findings of decreased Aov, Aovti, dv/dt_{max} and dv/dt_{mean} and increased acceleration times are also consistent with other reports about DCM (Gardin *et al* 1983). Both PEP (or PEP/√R-R) and the PEP:ET ratio data appeared to show minimal overlap with Normal Newfoundlands. A PEP:ET ratio exceeding 0.460 appeared to be reasonably sensitive (85.3%) and specific (82.9%) at identifying Newfoundlands with DCM.

There was no conclusive evidence of significant diastolic dysfunction in the DCM group compared with Normal dogs or Newfoundlands in the other categories, although trends to differences between Normal and DCM Newfoundlands were identified for a number of the mitral inflow and PVF parameters. Criteria for a diagnosis of impaired relaxation or restrictive physiology were not identified in the majority of these cases. Although the DCM group did have higher IVRT or IVRT/√R-R interval than the other groups, consistent with impaired relaxation, this difference did not achieve statistical significance.

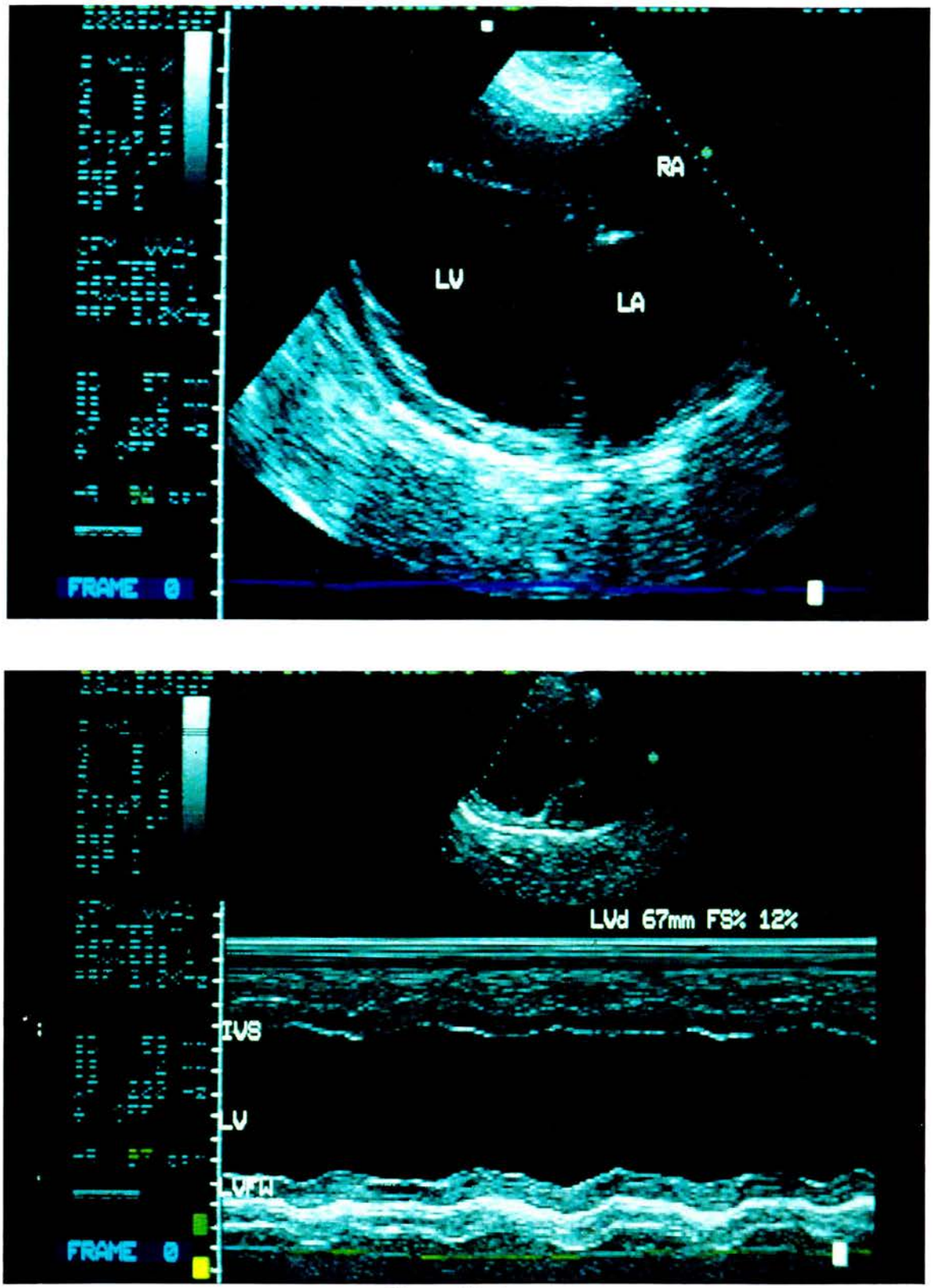


Figure B. 9 .

Newfoundland NF008/P534 with occult DCM showing marked left ventricular dilatation, but minimal proportional left atrial enlargement and marked hypokinesis. RPS 2D long axis view (top) and LV M-mode (bottom).

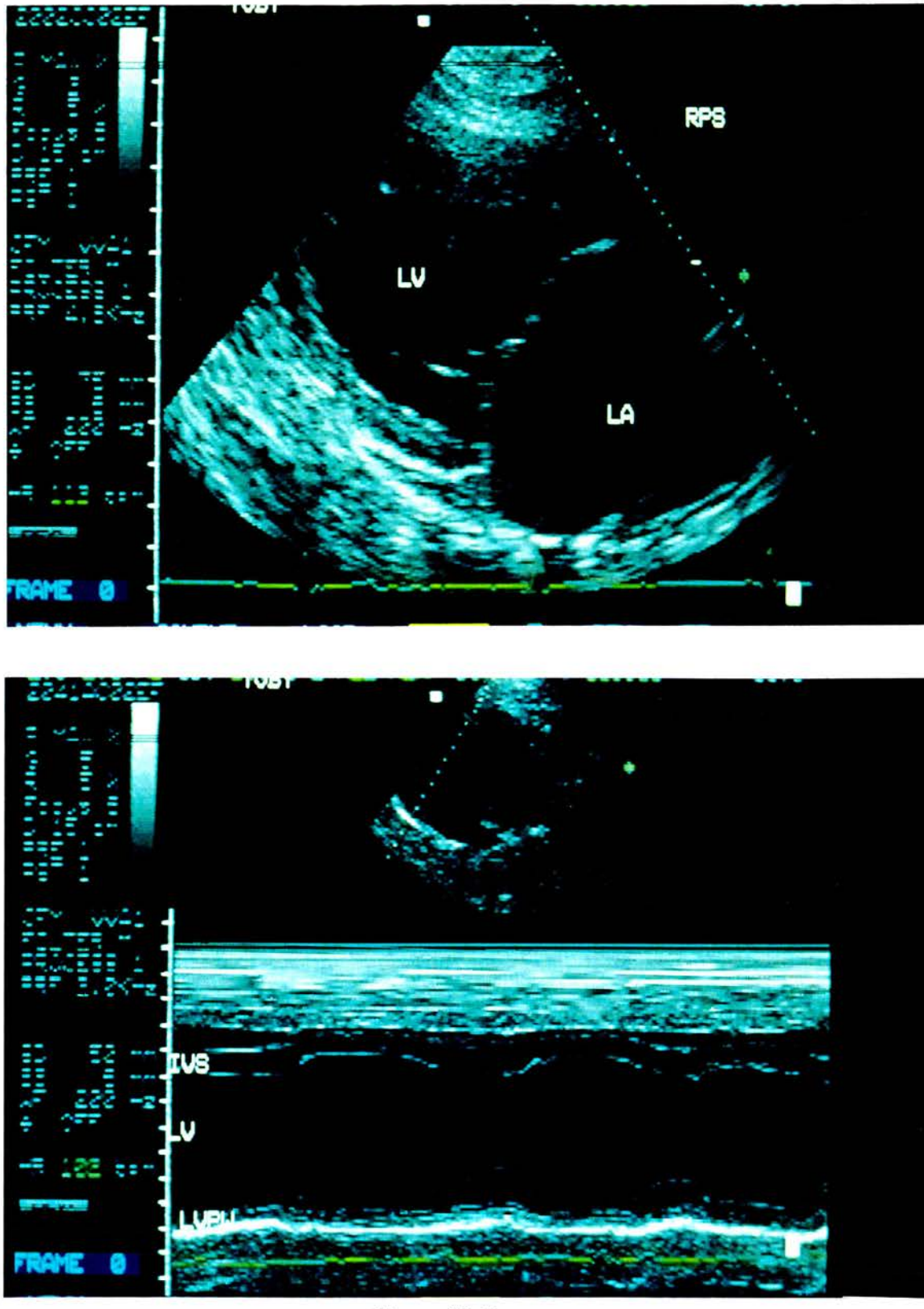


Figure B.10.

Newfoundland NF010/P10 with overt DCM, prior to any treatment, showing marked left ventricular dilatation, increased sphericity of the LV and left atrial enlargement. The left ventricle is very hypokinetic. RPS 2D long axis view (top) and LV M-mode (bottom).

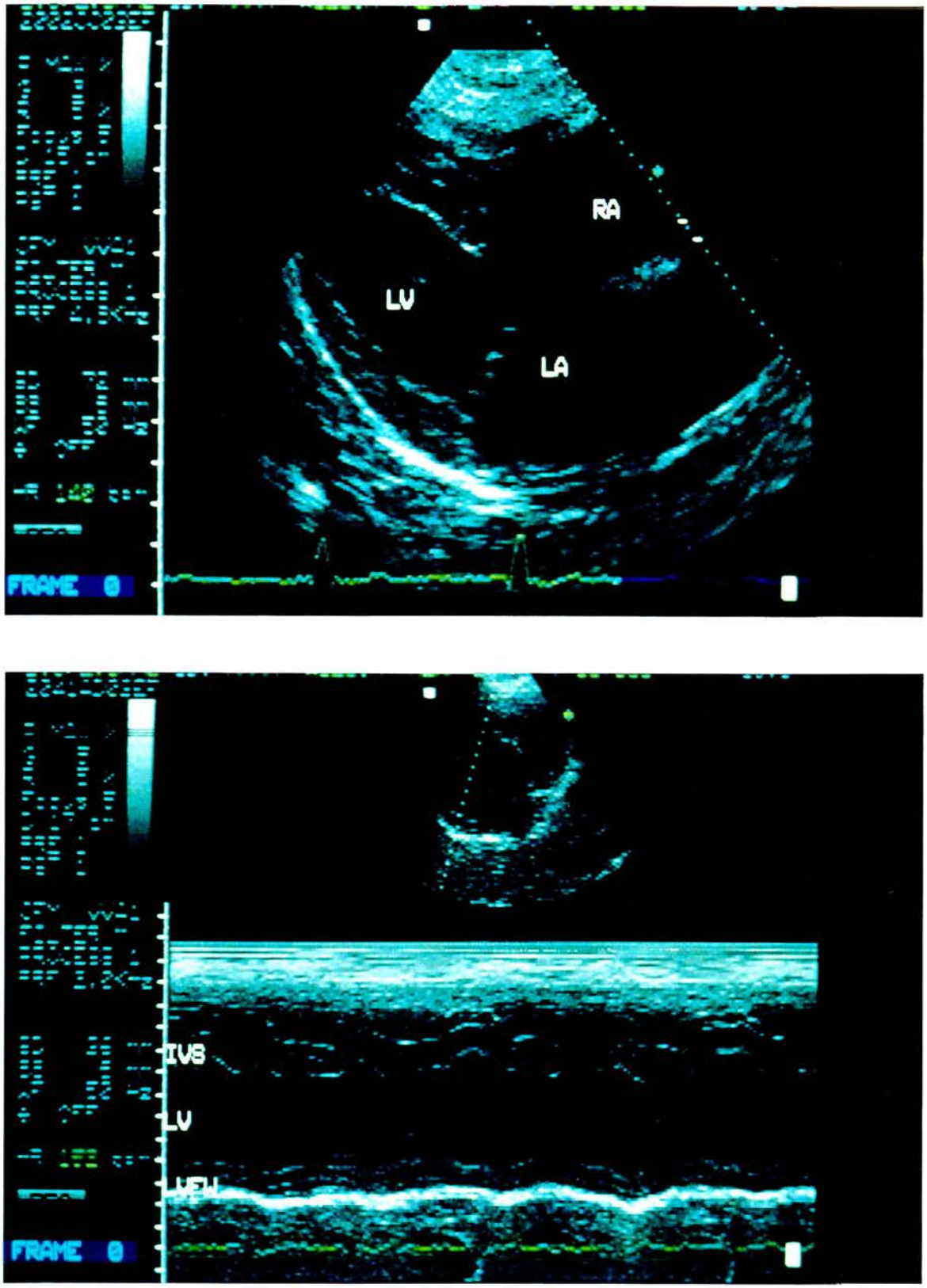


Figure B.11 .

Newfoundland NF002/P500 with overt DCM receiving treatment with an ACE inhibitor prior to diuretic or digoxin therapy. Although the LV is slightly rounded, it is not particularly dilated or hypokinetic. Marked biatrial enlargement is apparent. This dog was in atrial fibrillation. RPS 2D long axis view (top) and LV M-mode (bottom).

B.31. *Left ventricular enlargement category*

This was a small group, and even when differences appeared to exist in the box and whisker plots between this group and other groups, they often did not achieve statistical significance. These data showed that as well as the significantly increased M-mode LV diastolic dimensions, which had been used as a defining criterion in selecting this group, individuals had larger systolic diameters than the normal group. This group had a trend to larger LV diastolic volumes. Although M-mode diastolic wall thicknesses were not significantly different between groups, the fact that no eccentric hypertrophy was evident (from the LVpwd:LVIDd ratio) indicates that the chamber is significantly dilated and that wall stress can be inferred to be increased. This was surprising in that the left ventricular geometry and function was subjectively assessed as being unremarkable (Figure B.12.). The systolic wall thicknesses were increased compared with other groups for both IVSs and LVpws. It was striking that the conventionally used parameters of systolic function obtained by M-mode or 2D techniques were not depressed in this group; indeed individuals appeared to have enhanced systolic performance compared with other groups, although this did not achieve statistical significance for most of the parameters. Means for EF, SV or SVI, %thIVS and %thLVpw, FS and EF (Teicholz) all tended to be higher than the other groups. The Doppler parameters of aortic flow, dv/dt_{max} and dv/dt_{mean}, were not significantly different from the Normal dogs. It is possible that if these individuals do progress to develop DCM, at the stage of the recorded echocardiographic examination, they have suffered some myocardial insult, but are compensating by functioning further up the Frank-Starling curve.

The LAEI was depressed in this group in comparison with the other groups, which may indicate some intrinsic left atrial dysfunction.

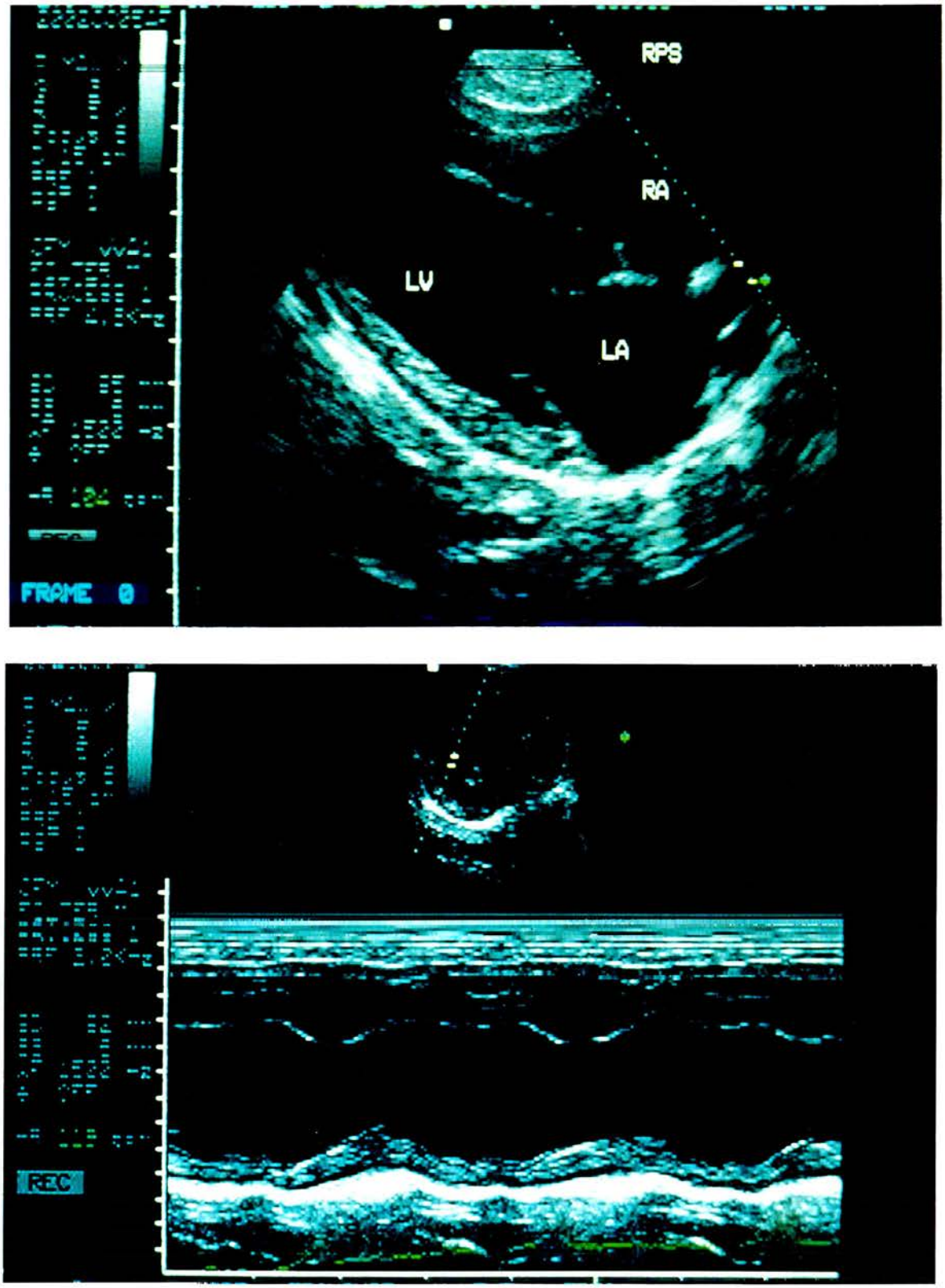


Figure B.12 .

Two dimensional RPS long axis four chamber view (top) and left ventricular M-mode (bottom) from a Newfoundland bitch in the LVE category (NF098/P73). This bitch was rescanned after 12 months, during writing of this thesis (data not included), and no significant changes were identified in M-mode dimensions.

The STIs, determined from subcostal Doppler aortic flow spectra, were more sensitive at distinguishing abnormalities of systolic function between the LVE group and Normal Newfoundlands. PEP or PEP/√R-R and PEP:ET ratio values were intermediate between the Normal and DCM categories, with similar values to both dFS. Differences were not identified as being statistically significant, probably because of the small numbers in the group. Aortic acceleration time and accel/√R-R also tended to be higher in the LVE group than Normal or SAS Newfoundlands, with similar values to both dFS categories.

Unfortunately, only two dogs in this group were able to be re-scanned at the time of writing. More individuals are required in this group, with further follow up in order to validate the tentative conclusion that these dogs do have early stages of myocardial disease. If confirmed, this group is consistent with the left ventricular enlargement category of human relatives of DCM patients described by Baig and colleagues (1998), which did appear to have progressive disease and this echocardiographic abnormality was concluded to be a manifestation of incipient occult DCM by these workers.

There was no evidence of significant diastolic dysfunction in the LVE group when this was compared with Normal dogs or Newfoundlands in other categories.

It was interesting to note that the recorded heart rate tended to be lower in the LVE group than the other groups during recording of many of the echocardiographic parameters, which was statistically significant in some instances. This may explain why some of the parameters of systolic function were apparently enhanced in this group, with greater time for diastolic filling. However, it does not support the fact that dogs in this group are compensating for some myocardial insult, when an increase in sympathetic drive and heart rate may be expected. However, it may be speculated that the left ventricular remodelling changes subsequent to such an insult have compensated sufficiently to preclude the necessity for such an increase in sympathetic drive. Other factors explaining both the left ventricular size increase and

relatively slow rates must, however, also be considered, such as concurrent hypothyroidism. This condition was not excluded in this study, although it is common in the Newfoundland breed. Fruhwald and colleagues (1997a) identified sonographic or subclinical functional abnormalities in 59 out of 61 human patients with DCM and they recommend that thyroid function is assessed in patients with DCM or atrial fibrillation. Calvert and others (1998c) found no difference in the incidence of hypothyroidism between normal or cardiomyopathic Dobermanns, another breed predisposed to this endocrinopathy.

As there was no gender influence on M-mode LV parameters in Normal Newfoundlands, the future criteria for the diagnosis of an LVE category should be modified. It should be based on body weight or BSA, and possibly also age, as these criteria all influenced the M-mode LV chamber parameters. The mean $\pm$ standard deviation for the LVIDd/m^2 for Normal Newfoundlands is 29.78 $\pm$ 3.4 mm/m^2. The upper limits may be defined as (mean +1sd) 33.18 mm/m^2 or (mean + 2sd) 36.58 mm/m^2. Weights or BSA were only available for six dogs in the existing LVE category. All six dogs were positive for LVE if the first cut off (mean +1sd) was used, but only two dogs were positive if the second cut off (mean +2sd) was used. If these cut-offs were applied to the Normal group, 9/64 dogs had LVIDd/m^2 > 33.18 mm/m^2 (14.1%) and only one dog had LVIDd/m^2 > 36.58 mm/m^2 (1.6%). In contrast, there were 13/26 dogs in the DCM group with LVIDd/m^2 > 33.18 mm/m^2 and 3/26 dogs in the DCM group with LVIDd/m^2 > 36.58 mm/m^2. It can be seen that these criteria are not particularly sensitive or specific and it is probably preferable to define a LVE category based on systolic dimensions indexed to BSA.

B.32. *Depressed fractional shortening categories*

Dogs in the two dFS categories (dFS<18% and dFS18-20%) tended to show this classifying feature due both to slightly smaller LV diastolic dimensions or volumes but also to increased LV M-mode systolic dimensions (although systolic volumes from 2D were similar to normal dogs). As well as FS, EF, SV and SVI were also reduced, with values intermediate between DCM and Normal groups. The LA parameters tended to be similar to Normal or even small, and the LAEI was intermediate between Normal and DCM groups, suggesting some degree of intrinsic left atrial dysfunction in this group. Although IVSd and LVpwd were similar for all groups, IVSs and LVpws for both dFS groups tended to be lower than Normal, and the %thIVS and %thLVpw were reduced compared with Normal dogs. The LVpwd:LVIDd index was similar to Normal dogs, suggesting that this group did not have increased left ventricular wall stress. They showed no alteration in LV chamber conformation, with normal or even slightly increased LVld:LVIDd measurements. Indeed, in some dogs, there was a subjective appearance of a long, thin left ventricle (Figure B.13.). The EPSS in these dFS groups was intermediate between Normal and DCM groups.

In the dFS<18% group, of the 29 scans, twelve dogs had not been re-evaluated at the time of writing. One factor identified to be responsible for the depressed FS% was the presence of regional wall motion abnormalities (e.g. Figure B.14.). Intercurrent disease may be responsible, such as lymphoma and hypercalcaemia in one bitch with two previously unremarkable scans, and laryngeal paralysis with cor pulmonale in another dog.

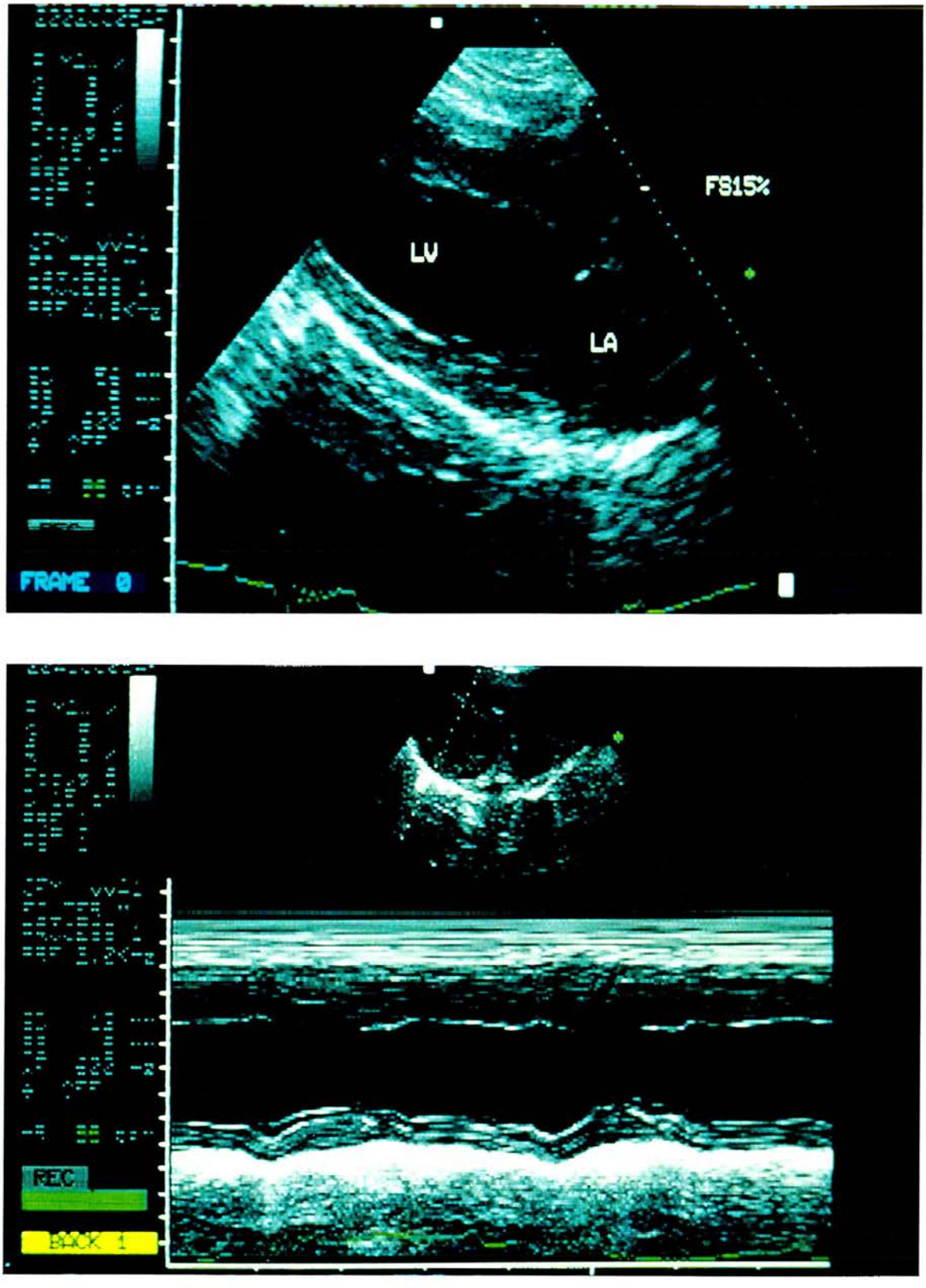


Figure B.13.

A young Newfoundland male from the dFS18-20% category (mean FS = 18.2%). This dog (NF099/P76) also had occasional VPCs during the echocardiographic / Doppler examination. A repeat scan has not yet been performed.

In the dFS<18% group, two dogs (litter mates) progressed to occult DCM by the follow up scan and one dog in the dFS18-20% category developed overt DCM with initially life threatening pulmonary oedema with ascites indicating biventricular failure, although he is currently stable. Dogs in the dFS<18% group included seven dogs with evidence of deteriorating systolic function. One dog improved marginally. The scans in the dFS18-20% probably do not represent a homogenous group. Almost certainly, some of these dogs are normal, but only serial evaluation will be able to separate normal from abnormal dogs. There were 24 scans in this group, five dogs of which have not received a repeat scan. One did develop DCM, as discussed above. Two dogs remained similar on repeat scan and three dogs showed improvement in the FS%, although two of these dogs had arrhythmias. Five dogs showed some deterioration in systolic function, although this was not always judged to be significant, although further conclusions cannot be drawn until future serial examinations are performed. Other dogs which deteriorated sufficiently to enter the dFS<18% group were discussed above.

Tidholm (1998) suggested that the echocardiographic finding of a fractional shortening of less that 25%, in the presence of radiographic or pathological evidence of congestive heart failure, was reliable in the diagnosis of DCM in approximately 90% of cases. However, no comment was made on the usefulness of depressed fractional shortening in dogs without congestive failure. In Tidholm's current longitudinal study of Newfoundland dogs in Sweden, some dogs with initially depressed fractional shortening are reported to normalise (Tidholm *et al* 1998b). In this study, dogs undergoing serial evaluation (see later), this was also recognised, although most dogs remained similar or progressed over the three years of this study.

In many cases, dFS appeared to be due to regional wall motion abnormalities. These could be very bizarre (Figure B.14.), including apparent paradoxical septal motion, but usually were due to either flat septal or posterior wall motion, or significant left ventricular free wall lag. The presence of these abnormalities makes it preferable to assess systolic function by methods other than M-mode. Paradoxical septal motion

has been recognised in veterinary cardiology since 1985 (De Madron *et al* 1985), although initially it was felt to merely indicate right ventricular volume or pressure overloads. In human cardiology, regional wall motion abnormalities are common, particularly with ischaemic heart disease but they may occur in cardiomyopathies also (Wallis *et al* 1984; Sunnerhagen *et al* 1990; Bach *et al* 1995). Segmental wall motion abnormalities are reported to be common. They tend to occur in older patients and are associated with less severe congestive failure, less LV dilatation and improved one year survival compared with patients with diffuse disease (Wallis *et al* 1984). Digitised ventriculograms have shown that diastolic regional wall motion abnormalities occur more frequently than systolic abnormalities (Sunnerhagen *et al* 1990), which was postulated to be due to a loss of myocardial elasticity in dilated ventricles. The apical segments were more commonly associated with regional wall motion abnormalities (Wallis *et al* 1984; Sunnerhagen *et al* 1990) and the proximal lateral wall shows relatively preserved function (Bach *et al* 1995). The reason for these segmental abnormalities in association with angiographically normal coronary arteries in DCM is unclear, although a proposed mechanism is that they are a consequence of heterogeneity of local wall stress (Sunnerhagen *et al* 1990; Bach *et al* 1995). Bach and colleagues (1995), however, showed that the regions with better function were associated with improved regional oxidative metabolism, assessed by carbon-11 acetate clearance positron emission tomography in an eight segment model of the left ventricle, suggesting that other mechanisms were also responsible for the heterogeneity of function.

In canine cardiology, coronary artery disease is presumed to be rare and is not excluded in the investigation of DCM, in the same way as coronary angiography is indicated in humans to rule out an ischaemic aetiology. However, a sibling of two DCM dogs in this study with echocardiographic features consistent with a diagnosis of DCM was shown to have a myocardial infarct at post-mortem examination (personal communication; Dr. P.R. Wotton).

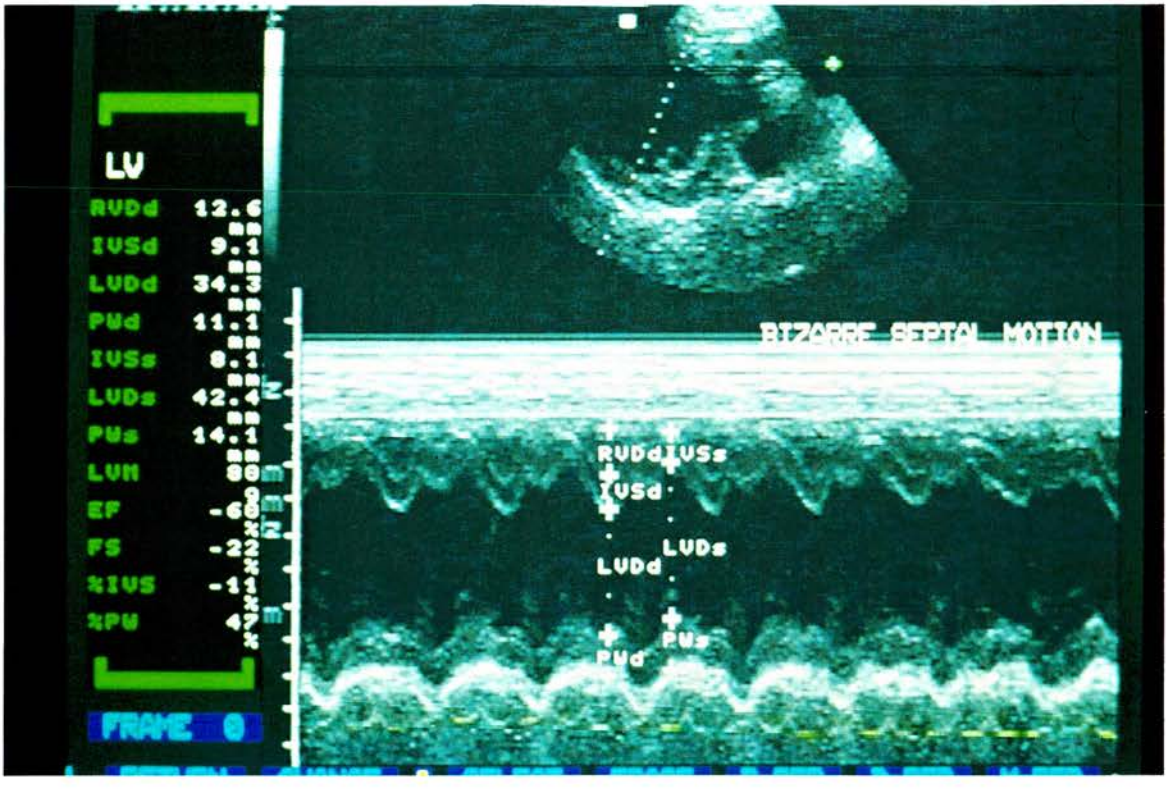


Figure B.14.1.

Bizarre septal motion from a Newfoundland from the dFS<18% category (NF038/P538). If the start of the QRS complex was used to prompt diastolic measurements, this corresponded to the nadir of the septal motion. Systolic dimensions of the LV were larger than the diastolic dimensions, with meaningless FS% derivative.

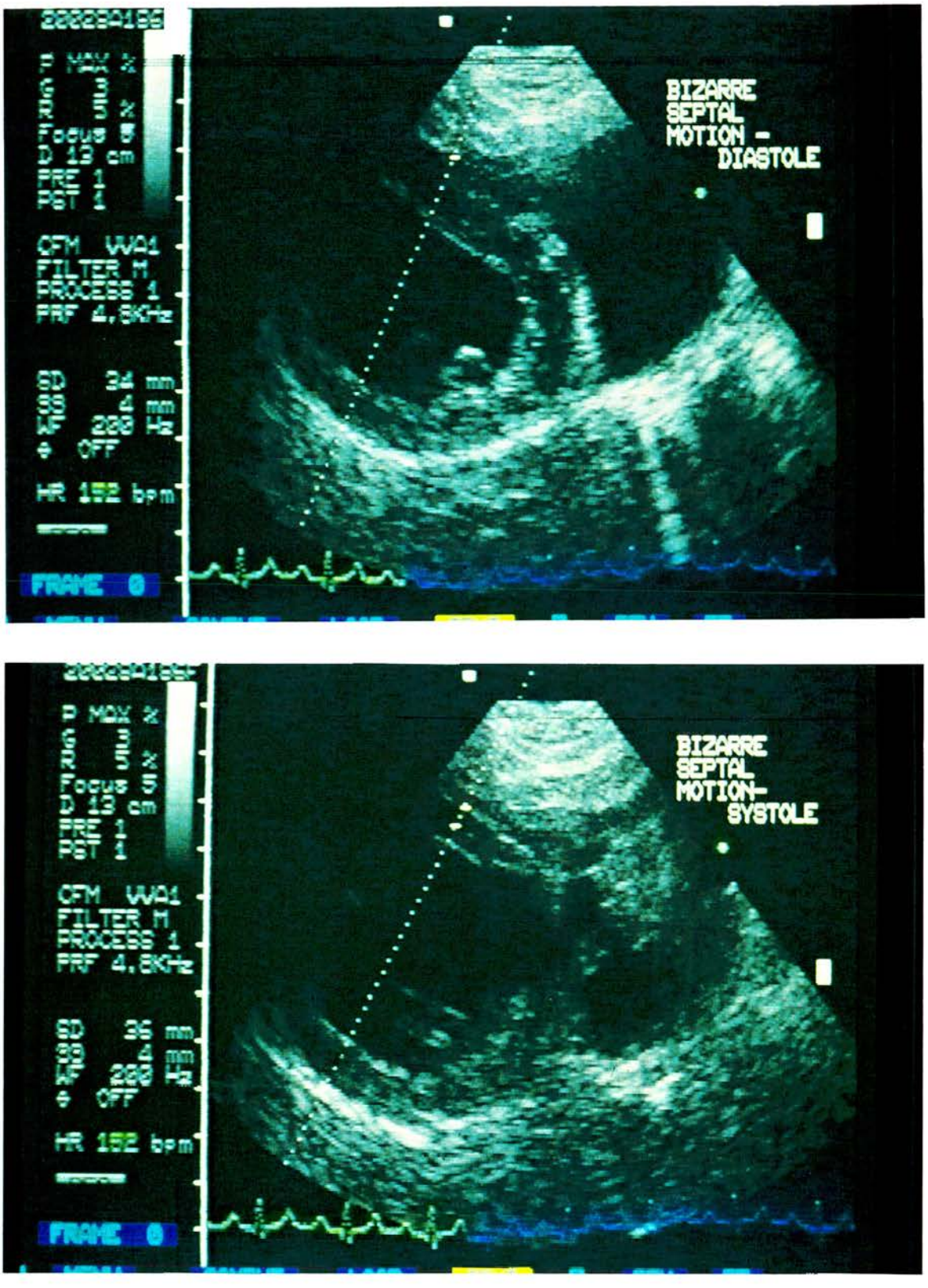


Figure B.14.2.

Two-dimensional RPS short axis views of the left ventricle analysed by frame by frame advancing of the videotape recording. Bizarre septal motion was recorded on the M-mode (Fig.**14**.1.) from a Newfoundland from the dFS<18% category (NF038/P538). If the start of the QRS complex was used to define diastole, the septum appears flat (top). After the T wave of the same cardiac cycle, representing systole, the septum has moved anteriorly rather than towards the left ventricular posterior wall (bottom), explaining the bizarre M-mode appearance.

It was interesting to note that the STIs determined from subcostal CW spectral Doppler of aortic flow did indicate a degree of systolic dysfunction between the dFS and Normal groups. This included Aovti, dv/dt_{max}, dv/dt_{mean}, PEP or PEP/√R-R, acceleration time or accel/√R-R and the PEP:ET ratio.

Most of the dogs in the dFS categories did not progress significantly or rapidly. Only three out of the total of 53 scans and 34 different individuals in these two groups have developed DCM, only one of whom is currently symptomatic. These data are consistent with the depressed fractional shortening group of relatives of DCM patients described by Baig and colleagues (1998). At the time of publication, most of these patients remained similar on repeat examination (within three years) and these workers suggested that depressed fractional shortening may be a limited manifestation of DCM in patients with familial disease. However, personal communication with one of the researchers in this group has indicated that some of these relatives have indeed developed DCM with longer follow up. Nevertheless, it appears that both humans and Newfoundland dogs with depressed fractional shortening deteriorate more slowly than dogs with left ventricular enlargement or dilatation or showing increased sphericity of the LV chamber.

In general, dogs in the two dFS categories did not show evidence of diastolic dysfunction, assessed by mitral inflow, PVF and IVRT.

B.33. *Newfoundlands with aortic velocities over 1.7 m/s or overt subaortic stenosis*

Forty dogs had peak aortic velocities (usually from a subcostal view) exceeding 1.7 m/s and were defined as subaortic stenosis (Lehmkuhl & Bonagura 1995). Of these, 23 had lesions evident by 2D echocardiography, such as echogenic nodules, or ridges (57.5%) (Figures B.15.1., B.16. & B.17.). Twenty six cases (65%) had turbulent blood flow assessed by CFDE determined presence of colour variance in the left ventricular outflow tract, often associated with the 2D lesions where these were imaged (Figures B.15.1., B.17.). CFDE showed that 27/40 dogs (67.5%) had concurrent aortic regurgitation (Figure B.17.). These findings support the fact that these dogs did have mild aortic stenosis (Lehmkuhl & Bonagura 1995).

The 2D echocardiographic findings recorded are entirely consistent with the pathology of discrete subaortic stenosis in the Newfoundland breed described by Pyle and others (1976). Pathologically, grade 1 SAS is recognisable on gross examination as one or more small slightly raised whitish nodules on the endocardial surface of the interventricular septum below the aortic valve. Grade 2 SAS is associated with a narrow ridge of whitish, thickened endocardium extending partially around the LVOT. In grade 3 SAS, a fibrous band or ridge completely encircles the LVOT. The fact that the subvalvular lesions are acquired after birth (Jones *et al* 1982) does not confound this study, where dogs were all over 18 months old.

In canine subaortic stenosis, the use of colour variance at the level of the obstruction has been described (Darke 1990;1992). Visualisation of the jet of aortic stenosis may be difficult (Khandheria *et al* 1986), and cranial RPS or LPS views are more likely to be successful than apical views, limited in adults (and therefore it is presumed in giant breeds of dog) by far-field imaging and the problem of attenuation of signal strength (Khandheria *et al* 1986). Although the use of green encoded pixels to identify dispersion or spectral broadening of the returning spectral Doppler signal has been traditionally considered to be evidence of turbulent flow, Perry (1989) pointed out that high velocity laminar flow, aliasing due to the Nyquist limit being exceeded,

may be misinterpreted by machine software as turbulent flow, and this author urged caution in interpreting the presence of turbulence from CFDE. Sahn (1988) went into considerable depth about the physics of displaying colour Doppler images, illustrating how an understanding of the physics was mandatory to interpretation of images. However, despite these possibilities of misinterpretation, the appearance of the colour variance map at the site of two dimensional subvalvular lesions (rather than at the "core" of LV outflow) is supportive of genuine turbulence, as illustrated in Figures B.15.1. and B.17.

A systolic heart murmur was detected in only 14/40 dogs (35%), with ten dogs with a grade 1/6 murmur, two dogs with a grade 2/6 murmur and one dog each with a grade 3/6 and a grade 4/6 murmur. It is striking that 26 of these dogs did not have an audible heart murmur at the time of auscultation, or even retrospectively, knowing the result of the Doppler velocity. The broad chest, thick hair coat and tendency to pant confounds the use of auscultation as a screening tool for aortic stenosis in this breed.

The aortic velocities, determined from the subcostal window, ranged from 1.71 to 2.15 m/s in dogs with no murmur detected (mean $\pm$ sd: 1.87 ± 0.14 m/s) (Figure B.15.2.). In dogs where a grade 1/6 murmur was detected, the aortic velocities ranged from 1.73 - 2.39 m/s (mean $\pm$ sd: 1.92 ± 0.19 m/s), and the two dogs with a grade 2/6 murmur had velocities of 1.92 and 1.79 m/s respectively. The aortic velocity in the old dog with the grade 3/6 murmur was 2.24 m/s and in the young dog with the grade 4/6 murmur was 3.94 m/s (Figure B.16.). No significant differences were identified between the groups divided by murmur grade on Kruskall-Wallis One Way ANOVA, although obviously data was limited in the higher grade murmur groups. An unpaired two-tailed *t*-test of the data in the murmur free and grade 1/6 murmur groups also failed to identify any significant difference between these groups. It appears that factors other than the simple velocity of peak aortic flow determine the audibility of the murmur and murmur grade. Many of these factors are dog related, with massive conformation, the tendency to being overweight, the very heavy coats

and the tendency to pant incessantly all confounding the use of auscultation. Operator determined factors may also have played a role, although this could not be investigated for this study and this breed without another cardiologist in attendance. These findings are a serious concern, when The Newfoundland Club utilises auscultation as a screening tool for aortic stenosis in this breed, with a cardiologist attending most of the major shows. It may be argued that for this particular breed, screening breeding stock by CFDE by an experienced echocardiographer is preferable.

The failure to demonstrate a significant relationship between any of the 2D or M-mode echocardiographic parameters and the peak aortic velocity obtained from the subcostal view was surprising. However, it probably just reflects the fact that the majority of dogs in the SAS group had only mild disease, as during the course of this study, participation of dogs with congenital heart murmurs had not been solicited.

The trend to thicker IVS or LVpw in the SAS group was predicted, as compensation for the increased afterload due to the presence of the stenosis. It was surprising to identify larger 2D end-diastolic volume index (EDVI) and a tendency to larger M-mode LV diameters in both diastole and systole in this group. However, Douglas and colleagues (1987b) described a sub-group of human patients with subaortic stenosis and reduced ejection fraction. These individuals were shown to have a larger LV cavity, lower relative wall thickness (i.e. LVpwd/LVIDd) and therefore higher circumferential wall stress than patients with subaortic stenosis with normal left ventricular function, despite a similar LV mass in both groups. The sub-group with lower ejection fraction also had altered LV geometry, with a more rounded LV cavity, as determined by the ratio between LV minor axis dimension and length. These authors concluded that the decreased pump function resulted in changes in LV shape and increased wall stress which made it impossible for LV architectural changes to normalise for increased afterload despite adequate LV mass (Douglas *et al* 1987b). It is possible that the general indication of low systolic function even in

normal Newfoundlands compared with published reference ranges for normal dogs resulted in similar changes occurring in Newfoundlands with aortic stenosis.

This group also tended to have larger left atria, presumably due to left atrial afterload being increased ejecting into a stiffer LV, even if LV diastolic pressure was not elevated. A trend to higher total LV stroke volume and stroke volume index was also identified.

Although the significantly higher Aov and Aovti was expected in an SAS group, it was striking that these dogs, with mild disease, showed significantly greater dv/dt_{max} and dv/dt_{mean} than other groups. Although this could be inferred to be a consequence of the higher peak velocity, the fact that the aortic flow acceleration time and accel/$\sqrt{}$R-R was lower than other groups indicates that this is genuinely due to enhanced left ventricular systolic performance compensating for the fixed stenosis and increased LV afterload.

Dogs with aortic velocities exceeding 1.7 m/s showed no conclusive evidence of diastolic dysfunction in this study, probably because the disease was mild in most cases. However, the group showed increased mitral Ev and Av and total vti than the other groups, in the absence of significant MR in the group. The SAS group also tended to have higher PVF velocities (Arv, Dv and R2v), and a higher total forward vti and Svti than the other groups, although ratios were unremarkable.

PAv and tricuspid Ev and Av were also increased compared with the other Newfoundland groups. The reason for increased velocities across all other anatomically normal valves as well as PVF is difficult to explain. It is interesting that the findings are not simply related to the left side. It is possible that generalised myocardial compensatory changes result in enhanced systolic performance as a consequence of the aortic stenosis. Without more detailed analysis of right sided function, or determination of cardiac output, further conclusions about these interesting observations cannot be drawn.

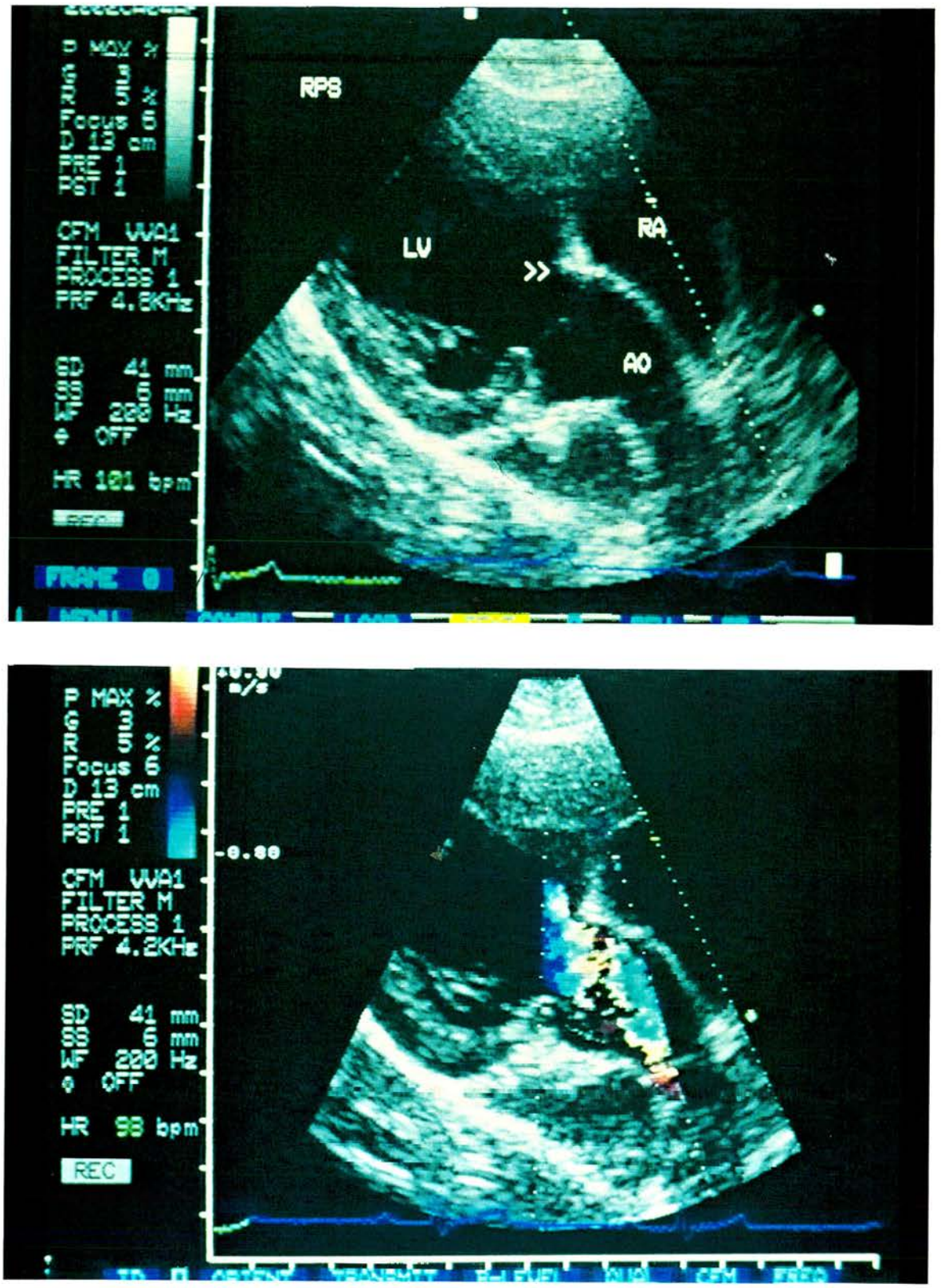


Figure B.15.1.

Two dimensional right parasternal cranial long axis view of the aorta and left ventricular outflow tract from a Newfoundland in the SAS group (Scan NF9/6). An echogenic nodule was evident below the aortic valve (top) and this was associated with colour variance (bottom). (Mean aortic velocity: 1.81 m/s.). This dog did not have an audible heart murmur.

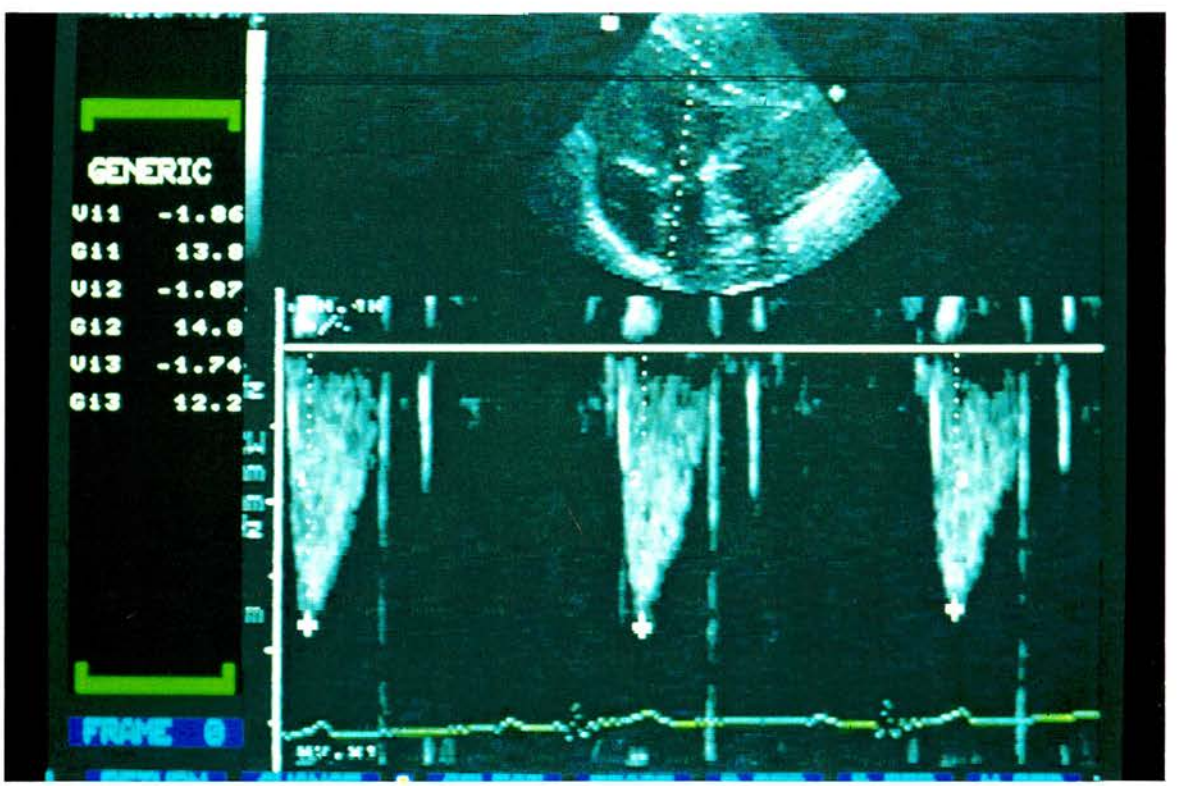


Figure B.15.2.

CW Doppler of aortic outflow from a subcostal view from a Newfoundland in the SAS group (Scan NF9/6), as in Fig. 15.1. The mean aortic velocity was 1.81 m/s, while very calm.

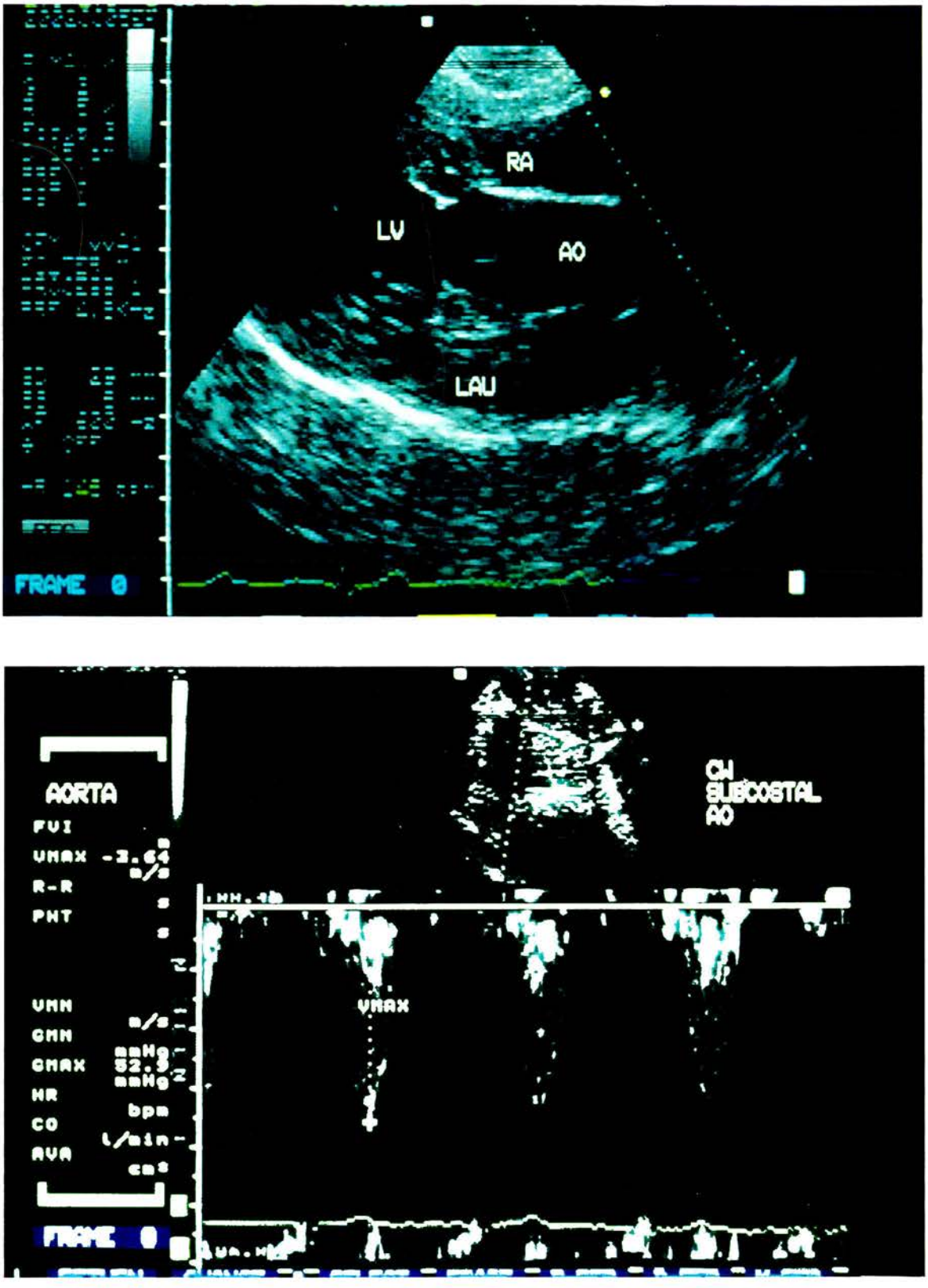


Figure B.16 .

RPS cranial long axis 2D view of the left ventricular outflow tract and aorta from a Newfoundland with subaortic stenosis of moderate severity (P301) (mean aortic velocity 3.94 m/s). A circumferential subvalvular ridge was identified (top). The bottom figure shows the subcostal CW Doppler spectrum from this case.

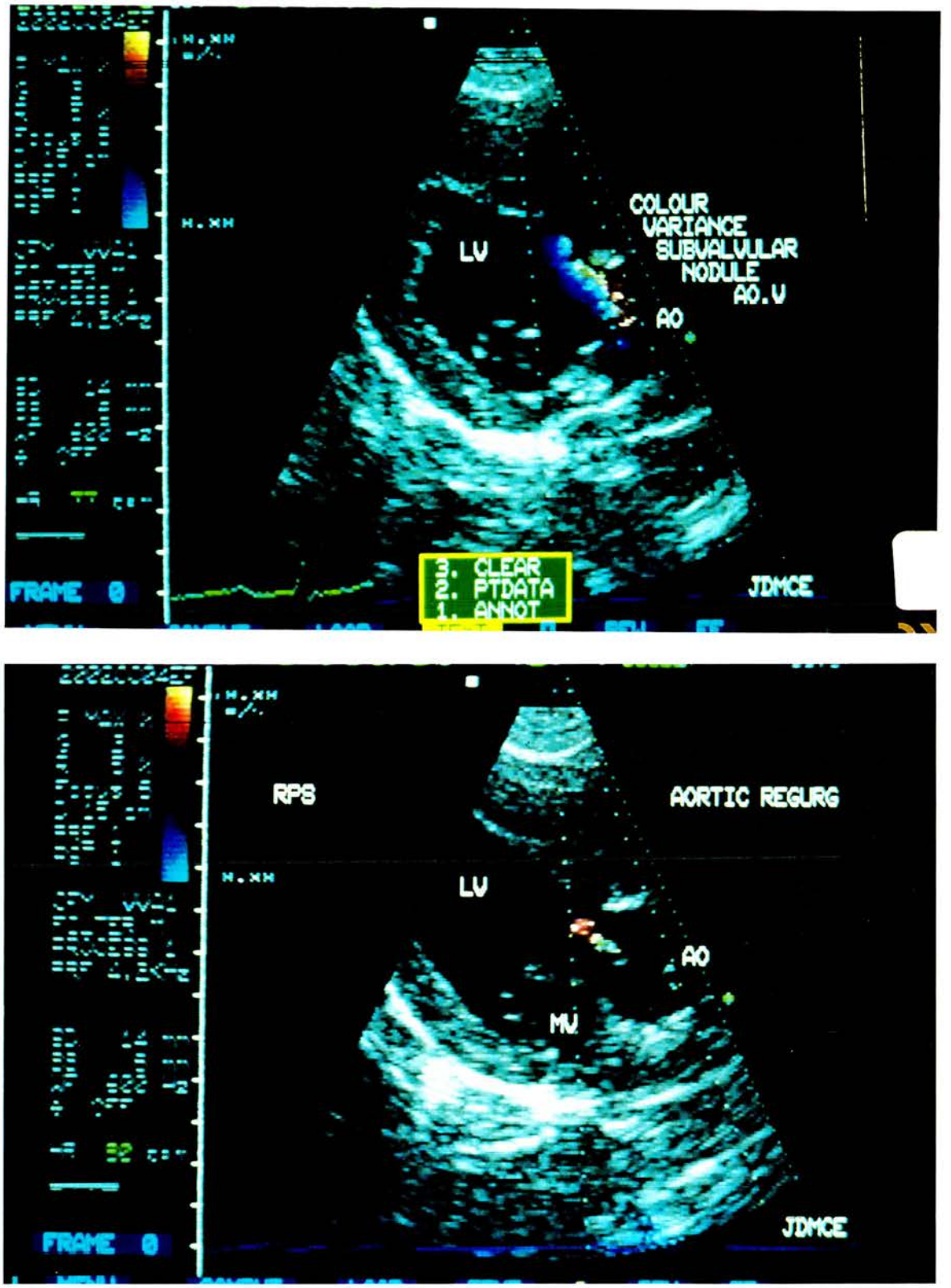


Figure B.17.

RPS cranial long axis 2D view and CFDE of the left ventricular outflow tract and aorta from a Newfoundland with mild subaortic stenosis (NF044/P285). Colour variance at the area of a distinct subvalvular nodule is shown (top). Mild aortic regurgitation was also apparent (bottom). The mean aortic velocity at presentation was 1.92 m/s (scan NF9/8) although on repeat examination, corresponding to these images, the mean aortic velocity was 1.52.m/s (scan 17/7, "Normal" category). The dog was systemically unwell and had shown considerable weight loss at the second examination.

B.34. *Other findings recorded*

There were two sudden deaths recorded during this study, both in dogs included in the Normal category. One bitch suddenly died, while exercising and eating cow-pats, and the owner considered that she had asphyxiated, although no post-mortem was carried out. Another sudden death, while running in a field, was recorded for a two year old dog. Although echocardiographic/Doppler evaluation was limited in this case, as the dog severely resented restraint, a full post-mortem examination by the dog's veterinary surgeon and a detailed cardiac examination by Dr. Rod Else failed to identify any significant abnormalities or the cause of death. A number of this dog's litter mates did show minor evidence of subaortic stenosis and the brief 2D and CFDE examination he received suggested that he had similar changes, consistent with the pathological grade 1 SAS described by Pyle and others (1976), although this was not confirmed by post-mortem examination. Consequently, caution in the interpretation of these two-dimensional echocardiographic abnormalities must be urged.

Other findings during this study included two dogs, with echocardiographic findings consistent with a persistent left cranial vena cava (Figure B.18.). One dog was not related to other dogs in this study, although he had other congenital defects, particularly affecting his urinogenital system. The other was related to other dogs in the study and he later developed evidence of occult DCM with atrial fibrillation.

Another congenital heart defect, an atrial septal defect, was identified in a dog included in the Normal category, as, other than mild right atrial enlargement, it did not appear to be haemodynamically significant.

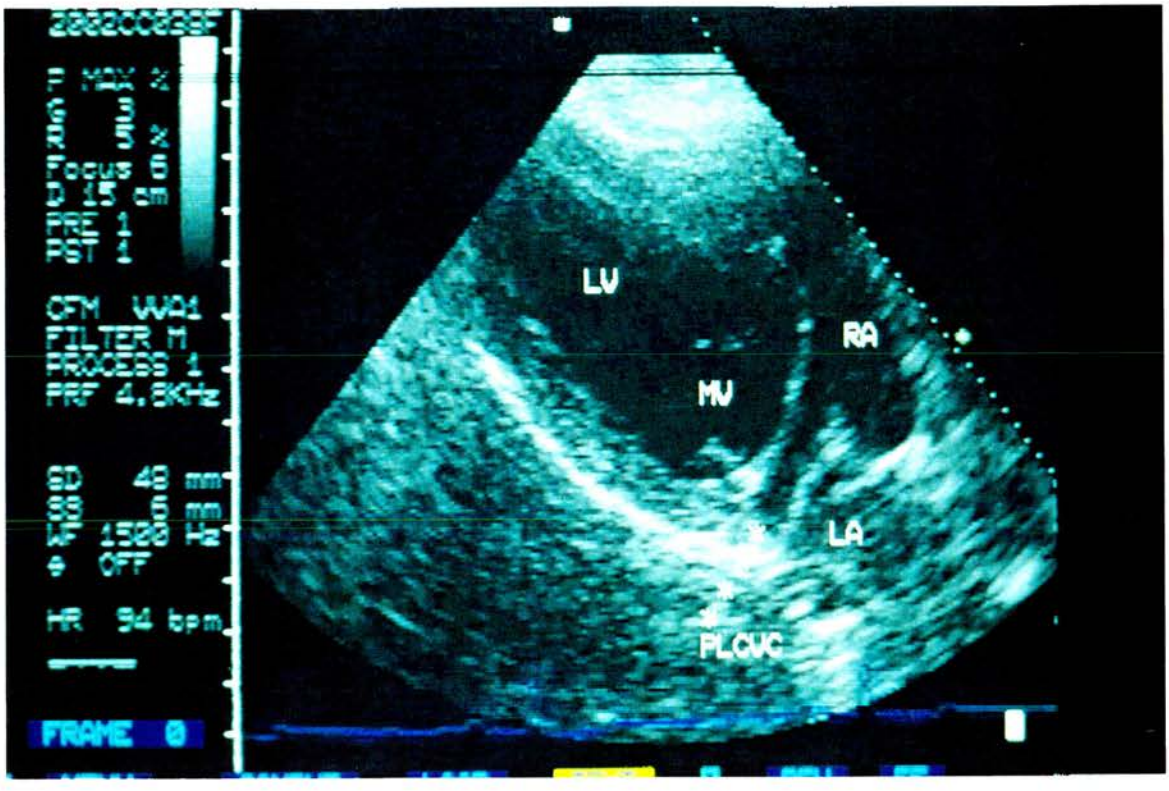


Figure B.18.

Right parasternal long axis view optimised to show a persistent left cranial vena cava entering the right atrium. Although "Normal" at the time of this scan, this dog (NF036/P40) did develop occult DCM with atrial fibrillation.

B.35. *Influence of independent variables on the echocardiographic parameters for the Newfoundland groups: Comparison with the Normal group*

Although a number of differences between the abnormal Newfoundland groups and Normal Newfoundlands were identified with the respect to the influences of independent variables on the 2D or M-mode echocardiographic or the Doppler parameters under investigation, it proved difficult to draw any conclusions about the meaning of such differences. The effect of these independent variables on echocardiographic parameters for the different Newfoundland groups is, however, detailed below.

B.35.1. *2D and M-mode parameters*

LVdv was positively and significantly related to BSA or weight in Normal dogs, the dFS<18% and SAS groups and more weakly with the DCM group, but not in the dFS18-20% or the LVE groups. Male gender had most influence over LVdv and EDVI in the dFS18-20% group. Age had a negative predictive influence on LVdv in Normal dogs and this was also evident in the dFS<18% group. No parameter significantly predicted LVdv or EDVI in the LVE group, although numbers were small.

LVsv was positively influenced by BSA or weight and negatively influenced by age in Normal dogs. Similar relationships to size were shown by the DCM, dFS<18%, dFS18-20% and the SAS groups. Only the SAS group showed a similar negative predictive relationship with advancing age as the Normal group. The LVE group showed that longer R-R intervals were associated with larger systolic volumes, which was surprising as one would expect improved systolic emptying of the LV with improved diastolic filling associated with longer cardiac cycles, by the Frank - Starling mechanism. Although the LVsv SAS group was predominantly predicted by BSA or weight, lengthening R-R interval was positively associated with larger volumes in a multiple linear regression model. The ESVI in Normal dogs was

significantly larger in males than females, even after normalising for BSA. Male dogs with DCM also had significantly larger ESVI than females.

EF was not significantly predicted by these independent variables in Normal, dFS18-20% or SAS groups. The major positive predictor of EF was the R-R interval in dogs with dFS<18%. The LVE group showed decreasing EF with advancing age. SV was positively associated with BSA or weight and negatively associated with advancing age in Normal and dFS<18% groups. The R-R interval predicted SV in dogs in dFS<18% and dFS18-20% groups.

In Normal dogs, LAad was positively influenced by age or BSA and R-R interval. BSA influenced LAad in the dFS<18% group, but age was negatively predictive of LAad in this group. In the SAS and dFS18-20% groups, LAad was positively influenced by size and also R-R interval. LAld positively associated with BSA or weight in Normal dogs, and dogs in the dFS<18% and dFS18-20% groups. LAld in the SAS group was positively influenced by the R-R interval. LAas and LAls were influenced by BSA or weight in Normal, SAS and dFS<18% groups and also weakly but significantly in the DCM group. LAls appeared to be significantly influenced by gender in the dFS18-20% group. 2D LAd was related to BSA or weight in Normal dogs, and the dFS<18% or dFS18-20% groups, although age was also a negative predictor of LAd in the dFS<18% and LVE groups and male gender a positive predictor of LAad in the dFS18-20% group. In the Normal and dFS18-20% groups, the major predictor of the 2D LAd:Aod ratio was the R-R interval. In dogs with dFS<18%, body size was predictive of the 2D LAd:Aod ratio. In association with the age related decline of LAd in the LVE group, the 2D LAd:Aod ratio also was significantly negatively associated with age. Rather bizarrely, the LAEI declined in larger dogs in the dFS18-20% group which is difficult to explain as in the other groups, it appears to be predicted by category rather than any independent variable.

LVIDd in Normal dogs was positively related to body weight or BSA and negatively predicted by age. These associations were also evident in the dFS<18% group.

LVIDd in DCM dogs was weakly correlated with body size. Dogs in the dFS18-20% and LVE groups also demonstrated the association between body size and LVIDd. In the dFS18-20% group, this parameter was also positively related to the R-R interval. In dogs with SAS, LVIDd was only weakly positively related to R-R interval (complementary to the effect of the R-R interval on the RVd measurement). No significant association between LVIDs and any independent variable was apparent in the Normal group, although this parameter was associated with size in the SAS group, the dFS<18% and the dFS18-20% groups and weakly so in the DCM group. The dFS18-20% also group showed a significant association between LVIDs and R-R interval. When LVIDd and LVIDs were indexed to BSA, both were negatively predicted by age in the Normal dogs. This was also evident for LVIDd/BSA in the dFS<18% group.

IVSd and IVSs showed no relationship with any independent variable in Normal dogs or dogs with dFS18-20% or SAS. In the DCM group, there was a trend to thinner IVSs/BSA in male dogs. The dFS<18% showed thinner IVSs in female dogs, although this gender influence was not apparent after indexing to BSA. IVSd in the LVE group was negatively predicted by R-R interval. The %thIVS increased with longer R-R interval in the dFS18-20% group.

LVpwd and LVpws were influenced by BSA or weight in Normal dogs. In DCM dogs, the only variable exerting an influence was the trend to increasing LVpwd/BSA with advancing age. No independent variable influenced the LVpw parameters in the dFS<18% group. In the dFS18-20% group, male Newfoundlands tended to have larger LVpwd and LVpws although the effect of gender was negated after indexing to BSA. In the LVE group, LVpws/BSA decreased in association with advancing age and %thLVpw also declined in older dogs. The LVpw parameters were not predicted by any of the independent variables tested in the SAS group. IVS and LVpw parameters are presumably predominantly affected by afterload rather than any of the independent variables in the SAS group.

LVpwd:LVIDd index was not influenced by any independent variable in Normal dogs, although in dogs with dFS<18% it appeared to be negatively influenced by body size and in dogs with SAS, it increased, presumably due to progressive concentric hypertrophy in association with advancing age.

The index of sphericity, LVLd:LVIDd, was not significantly influenced by any variable in Normal Newfoundlands or in any other group. The major influence on this parameter appeared to be category.

FS% and EF(Teicholz) were not influenced by any independent variable in the Normal group, the DCM group, the dFS18-20% group or the LVE group. However, in the dFS<18% group, these parameters were positively influenced by the R-R interval. This suggests that optimised diastolic filling will improve systolic function in this group. Rather surprisingly, in the SAS group, both FS and EF significantly decreased in larger dogs, negatively associated with weight or BSA.

In Normal dogs, mitral EPSS showed an association with body size, which was still apparent after normalising to LVIDd. EPSS/LVIDd significantly decreased in the dFS<18% group in association with longer R-R interval. R-R interval did not influence this parameter in Normal dogs. It may be inferred that EPSS declines with improved diastolic filling and more open mitral valve early in diastole with longer cardiac cycles.

M-mode LAs and Aod were both influenced by weight or BSA in normal dogs. Dogs with dFS<18% showed a predictive effect of size on LAs. Dogs with dFS18-20% only showed a significant positive influence of BSA or weight and age on Aod, although the effect of age was no longer apparent after indexing Aod to BSA. In contrast, the Aod in the LVE group appeared to significantly decline with increasing size, but it must be remembered that numbers were small in this category. Female dogs with SAS had larger Aod/BSA. The M-mode LAs:Aod ratio was not predicted by any independent variable in Normal dogs, although this ratio was greater in male

Newfoundlands with DCM than females. In dogs with DCM, LAs increased with advancing age. Aod was influenced by body size in the DCM group.

In Normal Newfoundlands, no significant independent variable influenced MAMseptal. This was also true in the DCM and dFS<18% groups. However, in the dFS18-20% group, a significant negative relationship was identified between MAMseptal and R-R interval. In contrast, the R-R interval was a significant positive predictor of MAMseptal in the SAS group. In the LVE group, significant positive predictors of MAMseptal were identified as R-R interval and body weight (or BSA) in a multiple linear regression model. MAMlateral was negatively influenced by age in Normal dogs and the dFS<18% and dFS18-20% groups. Body weight was the major significant predictor of MAMlateral in the DCM group and was also a positive predictor in the dFS18-20% group. When MAMmean was considered for Normal dogs, the R-R interval, age and gender all significantly influenced the parameter in a multiple linear regression model, although significant simple linear regression relationships were only identified for age (a negative predictor) and gender (larger values in male dogs). In the SAS group, similar influences of R-R interval and age were identified for MAMmean. MAMmean was not significantly influenced by any of the independent variables under consideration in the DCM, dFS<18%, dFS18-20% or the LVE groups.

B.35.2. *Doppler parameters of Aortic flow*

In Normal dogs, Aov tended to decline with lengthening R-R interval, and this relationship was also identified in the dFS18-20% group. Aovti tended to decline with advancing age in the DCM group, similar to the Normal Newfoundlands. The dv/dt_{max} was only significantly influenced by R-R interval in Normal Newfoundlands. However, in the LVE group, both age and gender appeared to be independent predictors of this parameter in a multiple linear regression model, with values tending to be negatively associated with age and the female gender. Aortic dv/dt_{mean} decreased in relation to longer R-R values in Normal Newfoundlands and dogs in the dFS18-20% group. Acceleration time was apparently significantly

predicted by gender in Normal Newfoundlands and the SAS group, with longer acceleration time in male dogs. This apparent gender influence in the SAS group was lost after indexing to √R-R, although it was retained in the Normal group. The LVE group showed a positive association between acceleration time and age, although this relationship was no longer apparent after indexing to √R-R.

PEP tended to increase with lengthening R-R interval in Normal dogs, and this relationship was also shown in the dFS18-20% group and the SAS group. Although a significant trend to longer PEP in male dogs in the Normal population, this relationship was not evident after indexing to √R-R and no gender influence was identified in the other Newfoundland groups. However, PEP did increase with advancing age in the dFS18-20% group and the LVE group, despite no evidence of age prediction on this parameter in the Normal group. However, the relationship between age and PEP was no longer apparent in the LVE group after indexing to √R-R.

The association of ET and R-R interval identified in Normal Newfoundlands was also evident in the dFS<18%, the dFS18-20% and the SAS groups. However, also influencing this parameter in the Normal group and both dFS categories was age (ET tended to decrease with advancing age) and gender in the dFS<18% group (ET tended to be greater in bitches). The influence of age was abolished for ET/√R-R in the Normal and dFS18-20% group.

The trend to increasing PEP:ET ratio in DCM dogs with advancing age was also present in the dFS18-20% and the LVE groups, although was not evident in the Normal Newfoundlands, and the age influence was no longer apparent in the LVE group after PEP:ET was indexed to √R-R. In Normal Newfoundlands, Vcf was not significantly influenced by age, but the DCM group and the dFS18-20% groups showed an age related increase in Vcf, which was difficult to explain. In contrast, the non-significant trend to a negative relationship between Vcf and advancing age became statistically significant for Vcf/√R-R and age in the SAS group. Vcf/√R-R

also appeared to be influenced by gender in the dFS18-20% group in a multiple linear regression model (with age), with a trend to higher Vcf in bitches, although this gender influence was not apparent in a simple linear regression model.

B.35.3. *Doppler parameters of mitral inflow*

In Normal Newfoundlands, mitral Ev, the E:A velocity ratio, Ev/total vti, Evti, E:A vti ratio and Evti/total vti were all significantly negatively influenced by age. Age was also one of the independent variables influencing the E:A velocity ratio in the dFS18-20% group in a multiple linear regression model with body weight and in the SAS group in a multiple linear regression model with R-R interval. Age was also found to be an independent predictor of Ev/total vti in the SAS group. In Normal Newfoundlands, mitral Avti/total vti was significantly positively associated with age. Normal Newfoundlands showed a positive relationship between age and E duration (and Edur/√R-R) and E deceleration time (and E decel/√R-R). Dogs in the SAS group also showed a significant relationship between E duration and deceleration time and age, although, in contrast to the Normal group, these relationships were no longer apparent after these parameters were indexed to √R-R interval. The SAS group showed a positive relationship of mitral A wave duration and Adur/√R-R with age.

In Normal Newfoundlands, body weight (or BSA) only significantly influenced Avti. However, in the DCM group, body weight appeared to be a significant predictor of a number of mitral inflow parameters. Ev:Av and Ev/total vti increased in heavier dogs and A duration (Adur/√R-R) were negatively associated with weight. Body weight also positively influenced Ev:Av in the dFS18-20% group. A similar relationship of E:A velocity ratio to body weight was also evident in the LVE group, although in a multiple linear regression model including R-R interval. In multiple linear regression models, increasing body weight was associated with a decrease in Av/total vti and Avti/total vti and an increase in Evti/total vti in the dFS18-20% group. Weight was a significant positive predictor of Evti/total vti and a negative predictor of Avti/total vti

in the SAS group. These relationships were in contrast to findings in the Normal group.

Mitral E:A velocity ratio was positively associated with R-R interval in Normal Newfoundlands and the SAS group. Evti and the E:A vti ratio were significantly associated with R-R interval in the SAS group. Av tended to decrease with lengthening R-R interval in Normal Newfoundlands and the SAS group, and this parameter, normalised to total mitral vti (Av/total vti), showed a similar relationship in the dFS18-20% and the SAS groups. Dogs in the dFS18-20% group also showed a significant positive relationship between the proportion of early LV filling (=Evti/total vti) and a significant negative relationship between the proportion of late LV filling (=Avti/total vti) and the R-R interval.

In Normal Newfoundlands, female gender was found to positively influence Avti and Avti/total vti. It negatively influenced the E:A vti ratio. In the dFS<18% group, a number of other predictive effects of gender were identified. Evti was significantly greater in male Newfoundlands and there was a non-significant trend to male dogs showing higher mitral E:A velocity ratio than females. Female dogs showed higher Av/total vti. E deceleration/√R-R was significantly longer in females in the DCM group, although no gender influence had been apparent on this parameter in Normal Newfoundlands or in the other Newfoundland groups.

In Normal Newfoundlands, E duration was significantly influenced by R-R interval as well as age. A similar positive relationship between E duration and R-R interval was identified in the dFS18-20% group. E deceleration time was significantly influenced by the R-R interval in Normal Newfoundlands. A duration was positively associated with R-R interval in Normal Newfoundlands, and the SAS and dFS<18% groups.

IVRT was positively related to age in the Normal group, but the other Newfoundland groups did not show any relationship of any independent variable on this parameter.

B.35.4. *Doppler parameters of pulmonary venous flow*

In Normal Newfoundlands, age significantly influenced a number of the PVF parameters. Arv, Sv, S:D velocity ratio, Svti, S:D vti ratio and the systolic fraction of total forward vti (=Svti/total vti) all increased with advancing age. The DCM group showed a similar but negative association of Sv and S:D vti ratio and age although these relationships did not achieve statistical significance. D duration (and Ddur/√R-R) significantly decreased with advancing age in the DCM group. D deceleration time and D decel/√R-R significantly increased with advancing age in the LVE group.

In Normal Newfoundlands, the S:D velocity and vti ratios and Svti/total vti all decreased with lengthening R-R intervals. Sv:Dv was also negatively associated with the R-R interval in the dFS18-20% and the LVE groups. R2 velocity tended to increase with longer R-R intervals in Normal dogs. S and D durations and D deceleration time were all positively associated with R-R interval. In the DCM group, Dv tended to decrease with lengthening R-R interval. R2 velocity increased with R-R interval, similar to the Normal group. In the dFS<18% and dFS18-20% groups, both Arv and Sv declined with lengthening R-R interval. A similar relationship was shown for Ar duration in the SAS group. The DCM group also confirmed the expected influence of R-R interval on S and D durations and D deceleration time, seen in Normal dogs. In the dFS18-20% and the SAS groups, R-R interval only significantly influenced D duration and D deceleration time. In the dFS<18% group, R-R interval only influenced D deceleration time.

Although gender did not influence any of the parameters of PVF in the Normal Newfoundlands, in the DCM group, sex influenced D deceleration time (and Ddecel/√R-R); values were longer in females. In the SAS group, in a multiple linear regression model, gender was shown to influence Dvti (with body weight), with a trend to higher Dvti in male dogs.

Body weight did not influence any of the PVF parameters in the Normal Newfoundlands. However, in the LVE group, Dv velocity was significantly positively influenced by body weight. Conversely, in the SAS group, Dv and Dvti were negatively associated with increasing body weight. The SAS group also showed a significant positive predictive influence of body weight on S duration and Sdur/√R-R. In the dFS18-20% group, R2 dur/√R-R was positively influenced by weight in a multiple linear regression model. It is difficult to explain the influence of body weight on these parameters, particularly when in some groups, it appears to exert opposing effects.

B.35.5. *Right sided function*

In Normal Newfoundlands, PAv tended to decline with lengthening R-R interval, and increasing age. In the DCM, dFS<18%, dFS18-20% and SAS groups, a similar negative association was identified for age, although this was evident only in the RPS recording of pulmonic flow in the DCM and dFS18-20% groups and the LPS view in the dFS<18% and SAS groups. The influence of R-R was not statistically significant in the other groups.

Tricuspid Ev and E:A velocity ratio decreased with advancing age in Normal dogs. The dFS<18% group also showed an age related decrease in E wave velocity, although the E:A velocity ratio was not significantly affected. The R-R interval negatively influenced the tricuspid Av and positively influenced the tricuspid E:A velocity ratio in Normal dogs. The DCM and SAS groups also showed an R-R interval determined decrease in Av although tricuspid Ev:Av was not significantly affected in the DCM group, although age influenced this ratio in the SAS group in a multiple linear regression model.

B.36. *Serial scans in the Newfoundland population*

Serial scans were undertaken in 54 dogs. When the serial scans were compared, a large number of differences were identified between the initial and second or final scan recorded for each individual, when the method of comparison used was the value of the percentage difference of the means and whether this exceeded the maximal value of the standard deviation expressed as a percentage of the mean for one of the scans. The Student *t* test also identified significant differences, and in general, as anticipated, these tended to identify the same parameters as the initial method. It may be argued that this technique in comparing scans is too conservative. Although it does take into account actual changes in the echocardiographic parameter under consideration, possibly as a consequence of evolving cardiac disease, it fails to consider day to day variation in the dog. Some dogs showed considerable changes between serial scans, in body weight, or heart rate between scans. If dogs became obese, it was much more difficult to obtain diagnostic images, or Doppler parameters with good signal:noise ratio. Even though the same operator and observer performed and measured all the serial scans, it is probable that there will be differences in technique and skill between the scans obtained at the beginning or towards the end of the study. Consequently, it was decided that a more valid method of comparing the serial scans was using the pooled coefficient of variation (c.o.v.) data from the boxer repeatability study. The fact that these data were generated from a different breed may be criticised. Boxers show more beat to beat variation as a result of accentuated sinus arrhythmia not generally evident in Newfoundlands. However, the quality of boxer echocardiographic images and the signal:noise ratio is generally better than in Newfoundlands, and the author presumes that an equivalent repeatability study in Newfoundland dogs would have resulted in greater coefficients of variation than boxers. Both operators and observers were very familiar with boxer echocardiographic images, whereas the second operator/observer in the repeatability study was far less experienced with Newfoundland images and this may also have led to a higher coefficient of variation in this breed. The day to day repeatability in the boxer study was over consecutive days, whereas in the Newfoundland study, the serial scans were many months apart. In conclusion, use of the boxer repeatability

c.o.v. data for comparison with the Newfoundland serial scans is valid, and probably is still fairly conservative.

B.36.1. *Normal Newfoundland category*

There were some marked differences between scans for the 2D echocardiographic LV parameters. Most marked was NF063/P244, where the initial LV volume was significantly smaller than the second scan. Since the LV length is small, it can be concluded that this is due to differences in imaging plane between the two scans. It is probable that the LV length and area was not maximised in the initial scan. Almost certainly, this was the greatest reason for differences between consecutive scans, as these parameters are exquisitely image plane dependent. Similar factors apply to the two dimensional echocardiographic criteria of the left atrium and aorta. Again, some marked differences between scans from the same individual are more likely to be due to a failure of regaining identical or correct imaging positions.

In general, the greater accuracy in obtaining M-mode echocardiographic images is reflected by the lower percentage differences between the means for these parameters in the Normal Newfoundland category. There were some marked differences identified, however, these could be explained by the presence of concurrent disease in two dogs (Dogs NF015/P42 and NF053/P229). Dog NF015/P42, with multicentric lymphoma at the time of her final scan, was nearly always a significant outlier in the data comparing serial scans regarding parameters of systolic function.

In general, results of percentage differences between scans for the same individual with respect to the Doppler parameters were more consistent with the c.o.v. data from the repeatability study. Unfortunately, where results were discordant, it is much more likely to be a consequence of image plane and angle dependence of these parameters.

In evaluating the serial scans from individuals judged to be normal, no attempt was made to correct for heart rate or any of the independent variables known to influence

various echocardiographic parameters in normal individuals. It is probable that if this had been done, less variability would have been evident.

In assessing whether differences between serial scans are genuine, further information is required. The individuals defined as being normal have to be confirmed to be normal with future repeat scans and preferably by post-mortem examination at the end of their natural lives. Future analysis should include all scans and not just the initial and final scan. Correction for changes in body condition, heart rate and probably age, where these are significant, should be made to allow valid serial comparisons to be made.

One general conclusion may be made, based on the serial scans from the Newfoundland group judged to be normal and the repeatability study. For most of the echocardiographic parameters under consideration, a difference between scans of more than twenty percent is likely to be significant, provided that both scans are of optimal diagnostic quality.

B.36.2. *Dilated cardiomyopathy category*

If this arbitrary figure of >20% was used to determine whether changes between serial scans were significant, general trends to increasing left ventricular volumes and left atrial size were identified in a number of the DCM Newfoundlands. The one dog who showed considerable echocardiographic improvement associated with optimising therapy (NF002/P500) showed a significant decrease in LV systolic volume.

Although the trends, achieving a significant difference exceeding 20%, of increasing M-mode left ventricular minor axis dimensions was expected with the progression of DCM, it was not evident in all dogs, and in NF002/P500 in particular, decreases were evident, presumed to be a consequence of successful treatment. It was surprising to find that in many of the DCM dogs, the IVS and LVpw (diastole and systole) increased in many of the dogs. This presumably represents an attempt at

compensatory hypertrophy in the early stages of this disease. Although FS% varied in either direction between sequential scans, it was unequivocally low. As expected, there was a trend to increasing mitral EPSS between scans in the same individual with progressive disease, although this could be reversed, presumed to be due to successful treatment. M-mode LAs decrease was in contrast to the 2D LA parameters, and this probably reflects the altered geometry of the LA and LV, making it difficult to maintain M-mode cursor position through the same part of the LA during sequential scans. No consistent change in the long axis contractility of the LV, assessed by MAM, was identified, although values tended to be low as expected in this DCM category.

The Doppler parameters of aortic flow in the DCM group indicate some differences, particularly in aortic velocity, which are not dependent on the underlying disease process, but rather reflect the importance of repeatable image acquisition and angle of interrogation for Doppler evaluation. However, these data indicate that the use of Doppler parameters and particularly STIs are extremely useful at indicating progression of disease. Only one dog showed an apparent improvement in PEP:ET ratio and shorter PEP (NF042/P542), but this individual was one with marked differences in recorded aortic velocity between scans and such differences may reflect variability in scan technique rather than genuine variation in this individual. The mitral inflow parameters do not show significant trends in any particular direction in association with disease progression with time in the DCM group. This is not unexpected, since there were few significant differences identified in the mitral inflow parameters when comparing Newfoundland groups. Similarly, no significant trends were identified in general for the DCM group with regard to the parameters of PVF. Although four dogs showed a decrease in Sv, these were not the same four dogs with an increased Dv, so it does not appear that the Sv:Dv ratio monitors progression of DCM.

Fewer differences of significance were identified for PAv from the LPS view than the RPS view, suggesting that the LPS view, as well as usually resulting in higher

velocities, is more repeatable. No general pattern was evident with progression of DCM over time with respect to the tricuspid inflow parameters.

B.36.3. *Depressed fractional shortening categories*

There were surprisingly few differences in the 2D LV or LA echocardiographic parameters between serial scans for most individuals in this final category. Again, M-mode criteria were surprisingly similar between scans, with few differences exceeding 20%. One peculiar exception was NF037/P48, who showed a dramatic decrease in LVIDd. Although this dog had gained six kilograms weight between the two scans, it is otherwise difficult to explain such a discordant result, unless it was measurement error (for example, the M-mode cursor in the initial measurement was obliquely positioned across the LV).

The Doppler parameters of aortic flow also showed few significant differences between sequential scans for the individuals in the dFS categories. Again, the trend to negative differences between the means for the corrected PEP:ET ratio in the majority of this group, which indicates larger values for the ratio for the repeat scans, supports the fact that this is a sensitive parameter for evolving deterioration in systolic function.

The Doppler parameters of mitral inflow failed to demonstrate any significant trends consistent with disease progression. In many cases, where there were significant decreases or increases in velocity or vti of E or A waves, these affected the same individual to a similar degree (e.g. NF006/P243), suggesting that such differences reflected different angle of interrogation or image position rather than genuine changes in mitral inflow. The PVF parameters for the dFS categories also showed surprisingly few significant differences between scans, and these probably reflect the repeatability issues rather than changes inherent in individual dogs. It is probable that the right sided parameters reflected differences between the images acquired and the angle of interrogation between scans, although there did appear to be a trend to decreasing PAv from either view in the later scans.

B.36.4. *Left ventricular enlargement category*

Although no significant differences greater than 20% were identified for the 2D LV echocardiographic parameters were recorded between the two scans for this individual, by the time of the second scan, the LV was assessed to be rounded, and the only reason the author was reluctant to identify this dog as occult DCM was the presence of a fractional shortening >20% and the absence of any supporting arrhythmia. The evidence of LAad increase supported the fact that this individual was probably developing DCM. The dramatic decrease in FS%, even though it remained above 22%, and increase in mitral EPSS adds further supportive evidence. The significant decreases in dv/dt_{max} and dv/dt_{mean} and Vcf and increase in acceleration time despite minimal differences in Aov or Aovti are also supportive of deteriorating systolic function. Mitral inflow parameters were probably not of great importance in determining disease evolution, although of note was the fact that the IVRT was >20% shorter than the initial scan despite similar heart rates.

B.36.5. *Category with aortic velocity >1.7 m/s*

Although some dogs were identified with significant increases in LV or LA parameters, it is difficult to separate genuine trends from a lack of optimal repeatability. There were very few differences identified between scans from the particular individuals with regard to the M-mode echocardiographic data. FS% significantly increased in two individuals, NF005/P515 and NF058/P256, both of which had depressed fractional shortening initially. Although significant differences in Aov were identified in this group, with associated significant differences in Aovti and dv/dt_{max} and dv/dt_{mean}, it is much more probable that the differences were due to inadequate alignment with aortic flow during at least one of these scans. Despite the depressed fractional shortening identified in two individuals on at least one occasion, the lack of progression of PEP:ET ratio suggests that these cases do not have progressive systolic dysfunction. No significant trends were identified for the differences between scans for the mitral inflow parameters or the pulmonary venous flow parameters.

In conclusion, it is apparent that it is difficult to distinguish between significant trends due to progression of cardiac disease and variability due to differing imaging planes and angles of interrogation. In general, differences have to exceed 20% to be presumed to be genuine rather than artefactual, although this varies between parameters, and the repeatability data from the boxer study (Appendix B.8.) forms a guide for values (coefficients of variation) which need to be exceeded before a genuine difference can be accepted.

Although serial evaluation of humans has been alluded to in evaluation of relatives of DCM patients (e.g. Baig *et al* 1998) and has been advocated in Dobermann dogs (O'Grady & Horne 1992;1998; Calvert 1995b; Calvert *et al* 1997b), there are no published reports giving the details of changes identified in humans or dogs.

SECTION C

THE GENETICS OF DILATED CARDIOMYOPATHY IN MAN AND DOGS AND PRELIMINARY WORK TO INVESTIGATE THE POTENTIAL FOR GENETIC LINKAGE ANALYSIS IN NEWFOUNDLAND DCM

A REVIEW OF THE LITERATURE

C.1. *Familial dilated cardiomyopathy in man*

Until recently, DCM in man was considered to be a sporadic disease (Fragola *et al* 1988; Valantine *et al* 1989; Michels *et al* 1992; Mestroni *et al* 1994a). Occasional reports of familial DCM were published as exceptions (Ross *et al* 1978; Voss *et al* 1984).

Currently, however, familial DCM (FDCM) is recognised to account for more than 20% cases of all cases of DCM (Michels *et al* 1992; Keeling & McKenna 1994; Mestroni *et al* 1994a;b; Goerss *et al* 1995; McMinn & Ross 1995; Mestroni *et al* 1995; Gavazzi *et al* 1998; Grünig *et al* 1998).

C.1.1. *The inheritance of human FDCM*

Human FDCM is most commonly transmitted as an autosomal dominant trait, (Gardner *et al* 1987; Schmidt *et al* 1988; Michels *et al* 1992; Keeling & McKenna 1994; Mestroni *et al* 1994a,; Durand *et al*, 1995; Keeling *et al* 1995; Krajinovic *et al* 1995; Mestroni *et al* 1995; Bowles *et al* 1996; Olson & Keating 1996; Messina *et al* 1997) although X-linked dominant (Mestroni *et al* 1994a), X-linked recessive (Neustein *et al* 1979; Valantine *et al* 1989), matrilineal (mitochondrial) (Zeviani *et al* 1991; Suomalainen *et al* 1992; Mestroni *et al* 1994a), autosomal recessive (McMinn & Ross 1995) and polygenic (Zachara *et al* 1993) modes of inheritance have been all been proposed in various families (Keeling & McKenna 1994; Mestroni *et al* 1995). It is now known that FDCM is not a homogenetic condition (Mestroni *et al* 1994a;b; 1995; Durand *et al* 1995). FDCM has proved to be a phenotypically (Grünig *et al* 1998) and genetically heterogeneous (Schultz *et al* 1995) disease.

Human FDCM is most commonly due to the presence of a single dominant locus (Michels *et al* 1992; Mestroni *et al* 1994a). The gene frequency is estimated to be 10^{-4} in the overall population (Mestroni *et al* 1994a; b). Because of the variable and histological heterogeneity of DCM in family members, incomplete penetrance is suspected. This may merely reflect an age related penetrance, with delayed age of onset, with penetrance increasing in older age groups (Mestroni *et al* 1994a). Keeling and others (1995) estimated that penetrance in their series of 40 families was variable and incomplete (65 - 95%) and the likelihood of an affected individual transmitting DCM to a child was 20%.

C.1.2. *Molecular genetics of human FDCM*

Cardiology as a discipline was initially slow to use the resources provided by the human genome project but recently genetic cardiology has been increasingly important (Schwartz 1994). A genome based resource is now available for molecular cardiovascular medicine, based on expressed sequence tags (ESTs) generated from human heart cDNA libraries (Hwang *et al* 1997). In identifying a genetic defect, traditionally, a biochemical defect leads to identification of the responsible gene, with demonstration of the mutation in this gene (the technique is known as *functional cloning*). In human FDCM, however, the underlying biochemical defect is unknown, and *reverse genetics* can be applied, or a *positional cloning strategy* (Marian & Roberts 1993). This technique detects linkage of the disease with an often anonymous chromosome locus, following which an attempt to identify genes encoded at that locus can be made. Initially these markers were restriction fragment length polymorphisms (RFLP) (Schwartz 1994) where 200 - 300 covered the human genome (Mestroni *et al* 1994a). More recently, hypervariable and highly polymorphic markers, amplified by the polymerase chain reaction (PCR) were described leading to accelerated identification of new loci for human diseases. These markers are short interspersed tandem repeats of nucleotides in dinucleotide, trinucleotide or tetranucleotide sequences which are distributed throughout the human genome, called microsatellite markers (Marian & Roberts 1993). High density linkage maps are

available for the human genome (Weissenbach *et al* 1992). Over 2000 microsatellite markers cover the human genome (Marian & Roberts 1993; Schwartz 1994).

The molecular genetics behind the technique of linkage analysis has been described for clinicians by Mestroni and colleagues (1994a;b; 1995). The aim is to correlate one of the known markers with transmission of the disease. Where they are linked, this implies that the disease gene and the marker lie in the same chromosome region. The probability that the genetic marker and the disease gene are linked is expressed as a LOD (logarithm (base10) of the odds) score. A LOD score exceeding +3 supports linkage with 95% confidence (=1:1000 odds for linkage) and a LOD score of less than -2 implies that there is no linkage (=1:100 odds against linkage). LOD scores vary as a function of θ, the recombination fraction, the frequency with which two loci recombine during meiosis. By calculating the frequency of the recombination events, the genetic distance between two loci can be estimated. If linkage exists with a recombination fraction, $\theta = 0$, the distance between the disease gene and the marker gene is close; there is tight linkage and the two genes are always inherited together. In contrast, a recombination fraction, $\theta = 0.5$ implies that the two loci segregate fully independently and there is no linkage (Mestroni *et al* 1994a).

The mapping of a chromosomal location of the disease gene can lead to study of this area with identification of the gene and its cloning with subsequent detection of markers responsible for the disease. For linkage studies to be successful, a large family is required, with members in several generations. The disease must be a homogenous trait with a known pattern of transmission and penetrance (Mestroni *et al* 1994a) and an accurate definition of the physical characteristics of the condition (phenotype) is required (McMinn & Ross 1995). In addition, the disease must be correctly diagnosed; an incorrect diagnosis will affect the interpretation of the linkage analysis (Marian & Roberts 1993). Indeterminate members may (Mestroni *et al* 1994b) or may not be included (Goerss *et al* 1995). With DCM, the variable penetrance of the disease, the absence of early clinical markers and premature

mortality is recognised to be a significant obstacle in collecting human families that are adequate for linkage analysis (Mestroni *et al* 1995). The lack of confirmed diagnostic criteria for the pre-clinical stages of DCM make phenotyping relatives difficult (Keeling & McKenna 1994; Baig *et al* 1998). All these factors confound the production of an informative pedigree suitable for linkage analysis.

C.1.3. *Human FDCM identified to date by Linkage Analysis*

Despite these difficulties, a range of chromosome loci have successfully been identified in some human families with DCM, as indicated in Table C.1. In most of these cases, a distinct phenotype, possible as part of a syndrome, has been identified.

TABLE C.1.
Chromosome loci linked with human dilated cardiomyopathy

Chromosome	Locus	Phenotype	Reference
Chromosome 1	1q32	DCM	Durand *et al* 1995
Chromosome 1	1p1-1q1	Conduction disease and DCM	Kass *et al* 1994
Chromosome 3	3p22 - q25	Conduction disease and DCM	Olson & Keating 1996.
Chromosome 6	6q23	Conduction disease, adult onset limb-girdle muscular dystrophy and DCM	Messina *et al* 1997.
Chromosome 9	9q13 - q22	DCM	Krajinovic *et al* 1995
Chromosome 10	10q21 - q23	DCM and mitral valve prolapse	Bowles *et al* 1996.
Chromosome X	Xp21	DCM with or without Duchenne / Becker muscular dystrophy	Towbin *et al* 1993 Muntoni *et al* 1993 Franz *et al* 1995 Muntoni *et al* 1997.
Chromosome X	Xq27-28.	DCM with or without Emery-Dreifuss muscular dystrophy	Hodgson *et al* 1986 Cartegni *et al* 1997
Chromosome X	Xq28	DCM with or without Barth syndrome, various X-linked infantile DCMs	Bolhuis *et al* 1991 Bione *et al* 1996 D'Adamo *et al* 1997.

C.1.3.1. *Autosomal loci*

Chromosome locus 1p1-1q1

Graber and colleagues (1986) described a phenotypically distinct condition characterised by arrhythmias associated with perturbed atrioventricular conduction which progressed to four chamber dilatation and impaired systolic function, with autosomal dominant transmission and high penetrance. Kass and colleagues (1994) identified the locus at the centromeric region of chromosome 1, with a maximal LOD

score of 6.28 at θ=0.1. A form of limb girdle muscular dystrophy (type 1B), has also been linked to this region (van der Kooi *et al* 1997) which has been proposed to be allelic (van der Kooi *et al* 1997; Messina *et al* 1997). The proposed candidate gene, encoding connexin 40, is also centromeric.

Chromosome locus 1q32

Durand and colleagues (1995) identified autosomal dominant DCM in a family of 46 members over four generations, with ten affected individuals. Two-point linkage analysis identified the locus at 1q32 with a highly significant LOD score of 6.37 at θ=0.

Chromosome locus 9q13 - q22

Krajinovic and colleagues (1995) detailed a large, Italian six generation kindred of eighty members, thirteen of who were identified with autosomal dominantly transmitted DCM. Linkage was established and multipoint linkage analysis identified the locus at 9q13-q22, with a peak LOD score of 4.2 at θ = 0.

Chromosome locus 10q21-q23

Familial autosomal dominant cardiomyopathy with mitral valve prolapse linked to chromosome 10 q21 - q23 was reported by Schultz and colleagues (1995) and Bowles and others (1996). A significant LOD score of 3.91 at θ=0 was obtained. Candidate genes in this region include muscle membrane proteins such as vinculin, metavinculin, actin, ankyrin and laminin, i.e. cytoskeletal proteins.

Chromosome locus 3p22 - q25

Olson and Keating (1996) described a family of 26 individuals, thirteen of whom were affected, with abnormal cardiac automaticity and conduction with progression resulting in dilated atria and / or ventricles with systolic failure and congestive heart failure. Transmission was autosomal dominant, with high penetrance. A LOD score of 6.09 at θ = 0 was achieved.

Chromosome locus 6q23

Messina and colleagues (1997) identified the locus associated with DCM, conduction system disease and an adult onset limb girdle muscular dystrophy in a large French Canadian kindred with autosomal dominant inheritance. Linkage analysis resulted in a LOD score of 4.99 at $\theta = 0$, linking the disease locus to chromosome 6 q23.

C.1.3.2. *X-linked DCM*

C.1.3.2.1. *Duchenne / Becker muscular dystrophy*

Dystrophin is the product of the Duchene / Becker muscular dystrophy gene. It is a large, sarcolemmal, cytoskeletal protein that forms an integral part of the oligomeric membrane associated protein complex known as the dystrophin - glycoprotein complex (Stevenson *et al* 1998). This complex tightly associates cytoplasmic, trans-membrane and extra-cellular components, and includes the four proteins of the sarcoglycan subcomplex, linked laterally to β–dystroglycan (fomerly called 43-DAG). β dystroglycan is bound to α–dystroglycan (a peripheral extracellular membrane protein), which is in turn is linked to laminin-2 (formerly known as merosin). The multiple protein interactions and the internal membrane cytoskeleton, mechanically coupled to the extracellular matrix, are strategically placed to strengthen the plasma membrane during muscle contraction (Stevenson *et al* 1998). Lack of dystrophin in Duchenne / Becker muscular dystrophy results in reduced capacity of the plasma membrane to withstand mechanical forces imposed by repeated muscle contraction, leading to damage, progressive necrosis and degeneration (Stevenson *et al* 1998).

Duchenne / Becker muscular dystrophy (MD) typically affecting young boys is associated with DCM and the cardiac involvement is frequently the cause of death in these patients (McMinn & Ross 1995). Duchenne and the milder Becker MDs are allelic disorders, both resulting from mutations of the dystrophin gene (Muntoni *et al* 1997). Myocardial involvement was found to be frequent in patients with subclinical or mild Becker's muscular dystrophy (Melacini *et al* 1996). It was postulated that the absence of significant myopathy led to such patients undertaking exercise, with the

resulting volume or pressure overload harming the dystrophin-deficient myocardium. Cardiomyopathy may be the only sign of dystrophinopathy in female carriers of Duchenne / Becker MD (Mirabella *et al* 1993; Nigro *et al* 1995; Politano *et al* 1996).

Additionally, a dominant X-linked DCM has been shown to occur unassociated with a skeletal myopathy, affecting males in their late teens, early 20s and females over 50. The disease is more severe and rapidly progressive in males than females (who are heterozygous) (Berko & Swift, 1987). Linkage analysis has identified linkage for X-linked DCM to Xp21, the dystrophin locus (Muntoni *et al* 1993; Towbin *et al* 1993). A deletion involving the first exon containing the muscle promoter of the dystrophin gene was identified in all affected patients by Muntoni and colleagues (1993) who speculated that dystrophin was relatively normal in skeletal muscle because transcription could be driven by another promoter, such as the brain promoter. Towbin and colleagues (1993) did not identify any deletion, but did suggest that in this form of FDCM without skeletal muscle involvement, the region of the 5' dystrophin coding sequence is affected, possibly involving a cardiac specific promoter which could lead to altering gene expression or abnormal splicing of the messenger RNA or to mutations in the amino terminus of the cardiac isoform of the protein. Other dystrophin mutations resulting in FDCM have been described (Franz *et al* 1995; Muntoni *et al* 1997; Ortiz-Lopez *et al* 1997; Ferlini *et al* 1998) showing that there is allelic heterogeneity of X-linked DCM (Ortiz-Lopez *et al* 1997).

C.1.3.2.2. *Emery-Dreifuss muscular dystrophy (EDMD)*

EDMD is an X-linked inherited disease characterised by early contracture of the elbows, Achilles tendons and post-cervical muscles, with slow progressive muscle wasting and weakness and cardiomyopathy, initially manifested as conduction disturbances Cartegni *et al* 1997). Cardiac problems are also recognised in female carriers. The disease was shown to be linked to the X chromosome, Xq28 (Hodgson *et al* 1986; Bione *et al* 1994). Bione and colleagues (1994) identified a novel gene, *STA*, with mutations associated with EDMD. The gene product is a ubiquitous protein, called Emerin, localised at the nuclear rim of most cell types. However,

Cartegni and colleagues (1997) found that in cardiomyocytes, emerin was specifically and uniquely localised to fasciae adherentes of intercalated discs and desmosomes and they proposed a role for emerin in membrane anchorage to the cytoskeleton. The fasciae adherentes anchor bundles of sarcomere myofilaments and consist of transmembrane adhesive glycoproteins, such as members of the cadherin family and cytoplasmic proteins like vinculin, catenins and actin binding proteins. A lack of cardiac emerin may alter cardiomyocyte adhesion and communication between adjacent cells resulting in arrhythmias and conduction disturbances (Cartegni *et al* 1997).

C.1.3.2.3. *Barth Syndrome (BTHS)*

Barth syndrome was first described in 1983 and is an X-linked cardioskeletal myopathy associated with growth retardation, neutropenia and abnormal mitochondria (Bione *et al* 1996). DCM is congenital and is associated with endocardial fibroelastosis, and is a common cause of death within the first months of life (D'Adamo *et al* 1997). There is a lipid myopathy with reduced plasma free carnitine. The locus for BTHS at Xq28 was identified (Bolhuis *et al* 1991). Gedeon and others (1995) reported that X linked fatal infantile cardiomyopathy also mapped to Xq28 and proposed that this condition was allelic with Barth syndrome. Subsequently, mutations in a novel X-linked gene, *G4.5*, were identified in BTHS patients and female carriers (Bione *et al* 1996). These authors proposed that the gene products, putative novel proteins, should be called tafazzins which were postulated to serve as a membrane anchor. It is now recognised that mutations in the *G4.5* gene are associated with BTHS, severe X-linked DCM and X-linked endocardial fibroelastosis, and therefore these conditions are allelic (D'Adamo *et al* 1997). Although most patients with X-linked DCM die within a few months of age, one patient was described who survived and was normal at 25 years old, which led the authors to consider that the tafazzins are important in foetal and neonatal life (D'Adamo *et al* 1997).

Another X-linked cardiac disease, isolated non-compaction of the left ventricular myocardium (INVM), characterised by pathognomic echocardiographic findings and post mortem changes (Chin *et al* 1990), has been linked to the *G4.5* gene at Xq28 (Bleyl *et al* 1997). The absence or inconsistent presence of other findings of BTHS in affected patients may suggest that the *G4.5* mutation affects a domain particularly important for cardiac muscle function (Bleyl *et al* 1997).

C.1.3.3. *Other genetic causes of FDCM as part of syndromes*

C.1.3.3.1. *Limb Girdle muscular dystrophies (LGMD)*

There are various types of LGMD which may have autosomal dominant or autosomal recessive inheritance in certain families, or be sporadic (van der Kooi *et al* 1998). The autosomal dominant LGMD1A and 1B are associated with loci at chromosome 5 and chromosome 1 respectively (van der Kooi *et al* 1997), but the gene products are not identified. Another locus for adult onset LGMD and autosomal DCM has been located by linkage analysis to chromosome 6q22-23, close to the laminin-α_2 gene (implicated in autosomal recessive congenital MD) although this gene was excluded, implying the presence of a second locus at this site (McNally *et al* 1997). LGMD1B, linked to 1q11 - q21, is frequently associated with cardiac involvement, initially as atrioventricular conduction disturbances and bradyarrhythmias, which may progress to DCM (van der Kooi *et al* 1997). It was speculated that this locus may be allelic to the autosomal dominant phenotypically distinct cardiomyopathy described by Graber and others (1986) linked to the centromeric region of chromosome 1 by Kass and colleagues (1994) (van der Kooi *et al* 1997). Autosomal recessive LGMD2 has also been linked to a number of different chromosome loci and the gene products have been identified in a number of cases. Some of these are the sarcoglycans, proteins which are part of the dystrophin - glycoprotein complex. Sarcoglycan is a complex of four transmembrane glycoproteins: a 50kDa (A2 or 50 DAG or adhelin, now called α–sarcoglycan), a 43 kDa (A3b, 43 DAG or β sarcoglycan), a 35kDa (A4, 35DAG or γ–sarcoglycan) and a 35kDa (δ–sarcoglycan) (Nigro *et al* 1997). LGMD2C is associated with mutations affecting the gene encoding γ-sarcoglycan (13q locus), LGMD2D with α-sarcoglycan (previously called adhelin) (17q locus),

LGMD2E with β-sarcoglycan (4q locus) and LGMD2F with mutations affecting δ-sarcoglycan located at 5q. There is a strong association between the absence of sarcoglycan and the presence of DCM (van der Kooi *et al* 1998). Deficiency of the 50kDa DAG, α-sarcoglycan or adhelin, was reported in the myocardium of a heart-transplanted patient with DCM and mild muscular dystrophy (Fadic *et al* 1996).

The small animal model for cardiomyopathy, the Syrian hamster BIO14.6, which has autosomal recessive cardiomyopathy (initially hypertrophy followed by dilatation), also has a mild skeletal muscle myopathy. Nigro and colleagues (1997) have recently identified the *cardiomyopathy* gene as the gene encoding δ-sarcoglycan, known to be located on hamster chromosome 9qa2.1-b1. In the hamster model, the δ-sarcoglycan regulatory sequences and the first exon is deleted and it shows that sarcoglycan mutations may result in predominantly myocardial expression and effects.

C.1.3.3.2. *Other myopathies*

Cardiac involvement has been described in a number of other myopathies. These include myotonic muscular dystrophy, with unstable repetition of a trinucleotide CGT sequence in the myotonin protein kinase gene (Phillips & Harper, 1997; Hayashi *et al* 1997; Hiromasa *et al* 1988), congenital MD, with deficiency of the laminin α_2 chain of merosin (McNally *et al* 1997; Spyrou *et al* 1998), proximal myotonic myopathy (Von zur Mühlen *et al* 1998) and others (De Visser *et al* 1992).

It may be appreciated that the myopathies and associated DCM are similar in that the genetic defects affect the cytoskeleton or cell to cell communication. Associated DCM differs from non-syndromic DCM in most cases by the initial presence of conduction disturbance or arrhythmias.

C.1.4. *Arrhythmogenic right ventricular cardiomyopathy / dysplasia (ARVD)*

ARVD is usually an autosomal dominant trait with reduced penetrance due to a degenerative process affecting right ventricular myocardium with focal necrosis followed by adipose and connective tissue replacement. Ventricular arrhythmias are common (Rampazzo *et al* 1997). The gold standard for diagnosis is the histological demonstration of a fatty or fibrofatty substitution of the right ventricular myocardium (Severini *et al* 1996). Over 30% of cases have a positive family history (Severini *et al* 1996).

ARVD is genetically heterogenous. Loci have been identified by linkage analysis at 14q23-q24 (ARVD1) (Rampazzo *et al* 1994), 1q42 -q43 (ARVD2) (Rampazzo *et al* 1995) and 14q12 - q22 (ARVD3) (Severini *et al* 1996) and 2q32.1-q32.3 (ARVD4) (Rampazzo *et al* 1997). An autosomal recessive syndrome called Naxos disease which is associated with right ventricular cardiomyopathy, diffuse nonepidermolytic keratoderma and woolly hair, limited to the geographical area of the Greek island of Naxos, has also been located by linkage analysis to a locus on chromosome 17 (q21) (Coonar *et al* 1998).

Rampazzo and colleagues (1994) noted that the degenerative process occurring in the myocardium was similar to that of skeletal muscle in Duchenne's muscular dystrophy. In the first identified locus, these authors noted that the gene encoding α–actinin-1 was located here, and they speculated that this could be a candidate gene, since there was strong structural homology between α-actinin and the N-terminal domain of dytrophin. Further support to the involvement of α-actinin was given when Rampazzo and colleagues (1995) identified the second ARVD2 locus at 1q42-q43; the location of the α-actinin-2 gene (*ACTN2*). Actinin genes are highly conserved throughout evolution. Sarcomeric α-actinin is the actin binding protein linking actin filaments from adjacent sarcomeres at the Z line (Arber *et al* 1997). However, in the new loci for ARVD3 and ARVD4, α-actinin genes were not known, although other candidate genes were proposed (Severini *et al* 1996; Rampazzo *et al* 1997).

C.1.5. *Mitochondrial cardiomyopathies.*

Structural changes in human mitochondrial DNA have been implicated in various diseases with dysfunctions in oxidative phosphorylation, called OX-PHOSPH diseases (Marin-Garcia *et al* 1996). Mitochondria have their own extra-chromosomal DNA which is double stranded and circular. It encodes for transfer and ribosomal RNA as well as proteins involved in cellular energy production (McMinn & Ross 1995). The human mitochondrial genome is 16.5 kb long and contains 37 genes encoding for 13 proteins involved in oxidative phosphorylation (Marian & Roberts 1996). Mutations in mitochondrial DNA are implicated in hypertrophic and dilated forms of cardiomyopathy (Ozawa 1995), usually also associated with neurological, myopathic and metabolic abnormalities. As mitochondrial DNA is inherited from the mother, mitochondrial mutation diseases are characterised by maternal inheritance (matrilineal). A number of mutations have been identified (Zeviani *et al* 1991; Hattori *et al* 1991; Obayashi *et al* 1992; Suomalainen *et al* 1992; Marin-Garcia *et al* 1996). Paternal transmission has been reported, explained by nuclear encoded mitochondrial proteins (Morgan *et al* 1996).

The heart in MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes) has been shown to have initial hypertrophy of the left ventricular posterior wall followed by progressive dilatation and deteriorating contractile function (Okajima *et al* 1998).

C.1.6. *Identification of candidate genes associated with DCM*

Although gene mutations affecting the dystrophin or *G4.5* genes have been identified and implicated some of the X-linked cardiomyopathies, progress in identifying the genetic defect underlying non-syndromic autosomal dominantly transmitted human DCM has been slow, despite intensive research on potential candidates, some proposed and some excluded (Kass *et al* 1994; Kelly & Strauss 1994; Krajinovic *et al* 1995; Schultz *et al* 1995; Leiden 1997; Messina *et al* 1997; Mestroni 1997). This was probably due to the small size of most families and confounding factors

including premature mortality, an absence of early markers of the disease and reduced penetrance (in part age-related) (Mestroni 1997). In addition, even when a locus is identified, the families are still too small for positional cloning of DCM genes (Olson *et al* 1998).

Recently however, cardiac actin gene (*ACTC*) mutations have been identified in DCM (Olson *et al* 1998). A candidate gene approach was used in two small DCM families, testing the hypothesis that actin dysfunction leads to heart failure. The cardiac actin gene, *ACTC*, is located on chromosome 15 (q14), an area not so far identified by linkage analysis in DCM families. Cardiac actin is the main component of the thin filament of the sarcomere. One end forms cross-bridges with myosin and the other end is immobilised, attached to a Z band or an intercalated disc. Actin consequently transmits force between adjacent sarcomeres and neighbouring myocytes to effect co-ordinated contraction of the heart. Olson and colleagues (1998) identified two missense mutations in the families, which affected amino acid sequences that are highly conserved. These mutations both affected the immobilised end of the actin filament, and resulted in altered rather than loss of function of actin. They proposed that DCM resulted from an episodic defect in force transmission, predisposing affected myocytes to mechanical injury and cumulative cell death, secondary interstitial cell fibrosis and cardiac dilatation, a degenerative process that may take years to develop.

This report supports the probability that DCM may result from defects in genes encoding cytoskeletal structure. It is apparent that a variety of defects lead to a similar phenotype.

Experimental models have also shown that DCM may result from cytoskeletal abnormalities. Leiden (1997) commented on the fact that one major type of gene implicated in DCM include those encoding structural proteins like dystrophin and other proteins of the dystrophin associated glycoprotein complex. These proteins organise the contractile apparatus of cardiac myocytes and ensure their structural

integrity. Another similar protein is muscle LIM (Lin-11, Isl-1 and Mec-3) protein (MLP). MLP may act as a scaffold protein to promote protein assembly along the actin based cytoskeleton (Arber *et al* 1997). Mutations in the cytoskeletal gene *MLP*, have been shown to cause cardiomyopathy in mice (Arber *et al* 1997). The human homologue of MLP(CLP) is located at chromosome 11p15.1, a locus not so far associated with human DCM (Arber *et al* 1997).

Juvenile transgenic mice with induced tropomodulin over-expression developed DCM with loss of myofibril organisation (Sussman *et al* 1998b). Tropomodulin is a tropomyosin binding protein that terminates the pointed end of actin filament polymerization. It is believed that tropomodulin:actin filament stoichiometry is critical for the maintenance of actin filament length (Sussman *et al* 1998a). Reduced tropomodulin content in vitro resulted in the formation of abnormally long actin filament bundles with resulting change and disruption of myofibril structure but not increased cell turnover. Regulated tropomodulin expression was concluded to be necessary to maintain stabilised actin structure within cardiomyocytes (Sussman *et al* 1998a).

Another hypothesis about the aetiology of DCM is through the function or perturbed function of cytokines. Cardiotrophin 1 (*CT-1*) belongs to a family of cytokines known to signal through transmembrane protein gp130 and to stimulate cardiomyocyte hypertrophy in vitro. Erdmann and colleagues (1997) used PCR single strand conformation polymorphism (PCR-SSCP) in 67 unrelated DCM patients and a group of controls. Two rare mutations were identified in DCM patients, giving rise to the hypothesis that cytokine encoding gene mutations may be important in the pathogenesis of DCM. Another transgenic mouse model of DCM was created by α MHC promoted over-expression of tumour necrosis factor α (TNFα) (Kubota *et al* 1997). It is known that increased TNFα in vivo results in a substantial decrease in cardiac contractility.

Another possible genetic influence over the cause or development of DCM include genetic factors linked to immunoregulatory loci. Carlquist and colleagues (1991) reported that HLA class II (DR and DQ) antigen associations were evident in DCM patients in a number of different studies and from different populations. They proposed that predisposing genetic factors may be present in DCM patients.

It can be appreciated that there is a vast range of potential candidate genes which may be responsible for the phenotype of DCM.

C.1.7. *Human hypertrophic cardiomyopathy: an illustration of a successful molecular genetic approach to cardiac disease*

Hypertrophic cardiomyopathy (HCM) in man has an autosomal dominant mode of transmission and is now known to be caused by a number of different chromosome loci and the genes so far identified in association with these loci all encode sarcomeric proteins (Marian & Roberts 1993;1995;1996; Anderson 1994; Hengstenberg & Schwartz 1994; Watkins *et al* 1995). Details of the molecular genetics of HCM are not directly pertinent to the subject of this thesis. However, it is worth noting that similar strategies in investigating FDCM in man and dogs should be successful.

All identified gene defects in HCM appear to result in abnormalities of the sarcomere, whereas in DCM, currently there is no single type of abnormality confirmed. The existence of phenocopies of DCM (e.g. ischaemic cardiomyopathy and alcoholic cardiomyopathy) have made the elucidation of FDCM more difficult than in HCM, as has the later age of onset and premature mortality associated with the disease. Both conditions pose problems in differentiating between normal relatives and relatives with early abnormalities. In HCM, the identification of relatives with genetic mutations similar to probands or other relatives with overt HCM has led to the realisation that echocardiographic identification of this disease is not precise (Solomon *et al* 1993). Hagège and colleagues (1998) screened genetically affected individuals with HCM with apparently normal hearts on echocardiography

and proposed that the phenotype of the disease was a continuous spectrum from normal structure to typical hypertrophy. Similar problems are encountered in the evaluation of FDCM.

C.1.8. *The diagnosis of DCM in asymptomatic relatives of DCM patients*

A diagnosis of DCM based purely on clinical symptoms is likely to significantly underestimate the prevalence of the disease. Echocardiography is a sensitive, non-invasive method of phenotyping the disease (McMinn & Ross 1995; Marian & Roberts 1996). In screening families to ascertain a familial prevalence, it is important to prospectively screen families of consecutive patients diagnosed with DCM using strict diagnostic criteria (Keeling & McKenna 1994; Keeling *et al* 1995). However, it is recognised that FDCM prevalence may be underestimated because some individuals remain asymptomatic and have no echocardiographic abnormalities for prolonged periods (McMinn & Ross, 1995). Screening relatives at one point in time is likely to lead to underestimation of the frequency of disease within a family (Goerss *et al* 1995). Csanády and colleagues (1995) reported that the time between onset of radiographic evidence of cardiomegaly and onset of clinical signs was on average 8 - 10 years and presumably this latent course will be even longer if echocardiography is used to serially assess relatives who eventually develop DCM. It can be seen that longitudinal screening is advisable (Lestuzzi *et al* 1991). The criteria for diagnosis of FDCM include the presence of DCM in two or more relatives (Mestroni *et al* 1994b) although Csanády and colleagues (1995) required that at least two individuals in at least two generations should be identified. The finding of one affected relative increased the probability that additional relatives in an affected family would be found (Keeling *et al* 1995).

Echocardiographic abnormalities are now appreciated to be common in relatives of DCM patients (Baig *et al* 1998; Gavazzi *et al* 1998). The problem of interpretation of these remains to be addressed (Lestuzzi *et al* 1991). Patients with left ventricular dilatation or slight hypokinesis are included in some series (Mestroni *et al* 1994b), classified as indeterminate (Mestroni *et al* 1994b) in other series and excluded in

others (Goerss *et al* 1995; Keeling *et al* 1995). Zachara and colleagues (1993) referred to their indeterminate members as "carriers" and defined these as asymptomatic relatives with echocardiographic or electrocardiographic abnormalities or both; they fully appreciated the importance in serial evaluation of such patients in accurately phenotyping these patients for pedigree analysis. Even where serial evaluation has been carried out to determine the progression of equivocal abnormalities, such studies have been short term (three years; Baig *et al* 1998). New diagnostic criteria are required for the diagnosis of early DCM (Mestroni 1997).

There are recognised limitations in family screening. DCM is a multi-causal disease and there is only very poor ability to distinguish between genetic and non-genetic disease (phenocopies). The disease itself shows marked clinical and morphological heterogeneity and the problem of identification of early disease all combine to confound the evaluation of a family (Lestuzzi *et al* 1991; Keeling *et al* 1995). However, family screening was felt to be advantageous by Keeling and McKenna (1994) and Keeling and colleagues (1995) who felt that newly identified individuals with DCM benefit from an early diagnosis as they benefit from early introduction of angiotensin converting enzyme inhibition therapy even while asymptomatic, based on data from the SOLVD trial (The SOLVD Investigators 1992). They also benefit from close monitoring. Increased survival of human DCM patients in recent years reflects earlier diagnosis and better treatment (Mestroni *et al* 1994b).

C.1.9. *Other genetic factors may influence the development of cardiovascular disease*

The genetic background can influence the manifestation of a disease. The major gene believed to influence the manifestation of a number of human cardiovascular conditions is the Angiotensin-1 Converting Enzyme (ACE) gene. This gene is located on human chromosome 17q23 (Mattei *et al* 1989). It is polymorphic based on the presence (insertion, I) or absence (deletion, D) of a 287 base pair *Alu* repeat sequence (Strachan & Read 1996a;b) within intron 16 (Rigat *et al* 1990; 1992), leading to three possible genotypes, DD, II and ID. The DD genotype is associated with increased

concentration of circulating angiotensin-1 converting enzyme (Rigat *et al* 1990; Tiret *et al* 1992).

The DD genotype has been associated with HCM (Marian *et al* 1993; Marian & Roberts 1996), myocardial infarction and artherosclerosis (Malik *et al* 1997) although this is controversial and such associations have been refuted (Lindpaintner *et al* 1996; Agerholm-Larsen *et al* 1997; Montgomery 1997). The association of ACE genotype and DCM is even less certain. Raynolds and colleagues (1993) reported that the DD genotype was significantly over-represented in patients with ischaemic and idiopathic DCM, although no such association was identified in by Montgomery and co-workers (1995) or Sanderson and others (1996). In DCM patients, the DD genotype is associated with increased mortality and higher plasma ACE activity than other genotypes (Skudicky *et al* 1998) although these authors showed that the DD genotype patients showed a greater improvement in left ventricular function when treated with an ACE inhibitor than other genotypes.

Zerjal and colleagues (1998) showed that certain variants of other genes involved in the renin-angiotensin system can represent susceptibility factors for the development of FDCM, including the angiotensinogen gene (AGT) M235T polymorphism and the gene for receptor 1 of angiotensin II (AT_1R) A1166C polymorphism.

Proto-oncogene expression may be important in the mechanism of hypertrophy. Kai and colleagues (1998) showed that c-H-*ras*, although normally expressed in cardiomyocytes, was correlated to cardiomyocyte diameter. Expression of *c-myc* mRNA is not normally evident in cardiomyocytes, but is identified in some HCM patients, in which cardiomyocyte diameter was larger compared with *c-myc* negative HCM patients. Further support for the role of proto-oncogenes in cardiac hypertrophy come from the cardiomyopathic hamster model. Hamsters with autosomal recessive cardiomyopathy may show cardiac hypertrophy (BIO14.6 and UMX6.1) or non-hypertrophy (TO-2). Although deficiency of δ-sarcoglycan results in reduced sarcolemmal sarcoglycan and dystroglycans in both forms, it has been shown that the

level of expression of *c-myc* which is upregulated only in the hypertrophic forms but not in the TO-2 strain (Sakamoto *et al* 1997). Thus the disease phenotype is due not only to the primary gene defect but also due to the expression of other genes.

C.2. *Canine dilated cardiomyopathy: is it a familial or an inherited disease ?*

It has been suspected for a number of years that canine DCM may be a familial or an inherited disease, mainly because of the specific breed predispositions (Lord, 1974; Bond & Tilley 1980; Tilley *et al* 1983; Fox 1988; Calvert 1992; Calvert 1995b; Sisson & Thomas 1995).

Boxers reported by Harpster (1983) were closely related and an inherited basis to the disease was suspected; some family lines had greater prevalence of the disease. Wotton (1998b) demonstrated familial disease in boxer cardiomyopathy, thought to be an autosomal recessive trait. Further evidence supporting a genetic basis to the disease was provided by Keene and others (1991), who documented that myocardial L-carnitine deficiency in a family of Boxers. Goodwin (1997) and Meurs and others (1998) thought that familial ventricular arrhythmias in three families of Boxers were inherited as an autosomal dominant trait.

Cobb and others (1996) showed statistically that there was an inherited basis to DCM in one line of Irish Wolfhounds, with an autosomal dominant mode of transmission. Wood (1983) felt that Doberman DCM may be familial and this has been shown by Meurs and colleagues (1996b). These latter authors are actively investigating familial disease in the Dobermann. Although some families of Dobermanns appear to have an autosomal dominant transmission of disease, with variable penetrance, not all families are consistent with this mode of inheritance and patterns have been difficult to determine (Meurs 1998).

An autosomal recessive mode of inheritance has been reported for the newly described juvenile Portuguese water dog dilated cardiomyopathy, affecting puppies from two to 32 weeks of age (Dambach *et al* 1999).

C.2.1. *Molecular Genetics applied to the dog and genetic diseases in the dog.*

Recently, there have been major advances in the development of a canine genome map. A large number of anonymous microsatellites (usually di- or tetra-nucleotide repeats) have been published (Ostrander *et al* 1992; Holmes *et al* 1993; Ostrander *et al* 1993; Holmes *et al* 1994; Mellersh *et al* 1994; Holmes *et al* 1995; Ostrander *et al* 1995; Francisco *et al* 1996; Mellersh *et al* 1997; Thomas *et al* 1997). There has been increased interest in developing the canine genome map, in part to obtain dog models for human disease (Ostrander & Giniger 1997).

The frequency of microsatellites in the canine genome varies with the size of the repeat. Dinucleotide (CA)n repeats occur approximately every 42 kilo-bases, whereas tetranucleotide repeats are present with 340 kilo-bases between loci (Rothuizen *et al* 1994), although some tetranucleotides such as (GAAA)n may be more common, estimated to occur every 100 - 200 kilobases in the canine genome (Francisco *et al* 1996). Although dinucleotide repeats are more frequent, there is rarely sufficient heterogeneity in a single dog breed for these markers to be informative for a linkage analysis study (Ostrander & Giniger 1997). Tetranucleotide repeats show greater polymorphism although they are more likely to mutate (Francisco *et al* 1996). Microsatellite markers ideally should have a large number of alleles in the general population (i.e. they should be polymorphic), and also in a specific breed and family, when investigating a genetic disease by linkage analysis. The polymorphic information content (PIC) indicates the informativeness of such a marker (Ostrander, 1998).

A number of microsatellites have been mapped separately by a predominantly European group, called DogMap (Lingaas *et al* 1997) and a group centred at the Fred Hutchinson Cancer Research Center in Seattle, US (Mellersh *et al* 1997). It has been decided that this latter map with 139 markers separated by average distance of 14.03 centiMorgans (cM) will be the foundation of a comprehensive canine genome linkage map (Mellersh *et al* 1997; American Kennel Club Molecular Genetics and Canine Genetic Health conference, St. Louis, 1997). To be useful, a genetic map should have markers placed at least every 10 cM. If the canine genome is of similar size to the human genome, this means that at least 350 informative markers are required (Ostrander 1998). So far, few genes have been added to the map and a physical canine map has been slow to evolve due to the large number of small chromosomes of similar appearance (a canine karyotype has only been agreed for 21 of the 38 pairs of canine autosomes) (Thomas *et al* 1997).

However, physical mapping in association with the developments in the linkage map is under rapid development by a number of groups using different techniques. These include: gene specific universal mammalian sequence tagged sites (Venta *et al* 1996); anchor loci from comparative mammalian mapping and identification of sytenic homology in the dog to assist in developing a functional genetic map (Meera Khan *et al* 1984; Sack *et al* 1996; Werner *et al* 1997); the use of fluorescence in situ hybridisation (FISH) techniques (Dutra *et al* 1996; Fischer *et al* 1996; Guevara-Fujita *et al* 1996; Thomas *et al* 1997); chromosome specific paints from a high resolution flow canine karyotype (Langford *et al* 1996); the use of comparative anchor tagged sequences (CATS) (Lyons *et al* 1997); and construction of canine-rodent hybrid cell lines (Langston *et al* 1997). The rapid evolution of a canine physical map offers opportunities for multipoint linkage analysis and a search for candidate genes.

Interbreed variability is also under investigation (Fredholm & Winterø 1995; Ezer *et al* 1996; Pihkanen *et al* 1996; Zajc *et al* 1997). Additionally, studies on the homology between the human, mouse, canine and other mammalian genomes are in progress and will allow identification of regions of comparative interest (Dutra *et al*

1996; Venta *et al* 1996; Mellersh *et al* 1997; Werner *et al* 1997). The degree of identity between canine and other mammalian index species sequences for certain genes is between 70 - 100% (Venta *et al* 1996).

To date, canine microsatellites have been used for dog parentage testing (Binns *et al* 1995), linkage analysis in Copper toxicosis in Bedlington terriers (Yuzbasiyan-Gurkan *et* al 1997; Holmes *et al* 1998a) and progressive retinal atrophy in miniature long-haired Dachshunds (Ryder *et al* 1998), and a specific allele of a microsatellite marker has provisionally been linked to absence of von Willebrand's disease status in Dobermanns (Holmes *et al* 1996).

Specific gene defects have also been identified, including the genetic basis of fucosidosis in English springer spaniels (Occhiodoro & Anson 1996) which has resulted in development a DNA test for this disease and for the carrier state (Holmes *et al* 1998b).

An interest in the genetics of certain conditions is blossoming in veterinary cardiology (Towbin, 1996a; 1996b; Meurs 1997). The modes of inheritance of various canine congenital heart disease has been investigated (Patterson 1996). This includes subaortic stenosis in Newfoundlands, which is now believed to be an autosomal dominant condition with variable expression (Patterson 1996) after initial doubt (Pyle *et al* 1976). Indeed, many conditions in man and dogs initially felt to be polygenic or multifactorial (Robinson 1991) are now believed to have more simple inheritance (Patterson 1996). The conotruncal defects affecting Keeshonden, initially thought to be have a polygenetic basis, is now known to be associated with a single gene defect in homozygous animals, with incomplete penetrance in heterozygotes (Paterson *et al* 1993).

Kittleson and colleagues (1998) have reported on an autosomal dominantly transmitted hypertrophic cardiomyopathy in Maine coon cats. The gene defect has not yet been identified.

C.2.2. *Canine DCM: Molecular genetics*

Although a number of groups are interested in this area, relatively little has been published. Schatzberg and colleagues (1998) reported on a massive deletion of all three dystrophin promoters (cortical, purkinje and muscle) along the entire length of the dystrophin gene in two male German short-haired pointer littermates with severe skeletal myopathy and DCM. Schatzberg and co-authors (1997) found no abnormality in any of the three dystrophin promoter regions in five male Dobermanns with DCM.

Western blot analysis was used by Spier and colleagues (1998) to assess the presence of the protein products of the DCM candidate genes, dystrophin, alpha-sarcoglycan and beta-dystroglycan, in the myocardial tissue from six dogs with DCM (4 Dobermanns, one Irish Wolfhound and a Boxer) and six control dogs. No abnormalities were detected in these proteins in these samples.

PRELIMINARY WORK TO INVESTIGATE THE POTENTIAL FOR GENETIC LINKAGE ANALYSIS IN NEWFOUNDLAND FAMILIAL DCM

MATERIALS AND METHODS

C.3. *Aims of this study*

It was evident during investigation of the Newfoundland population that DCM was a familial disease. An aim of this part of the study was to determine the mode of inheritance of DCM within this breed.

The extended family of Newfoundland dogs investigated as the major part of the Newfoundland population in this study appears to be suitable for a linkage analysis study. It is large, has individuals in several generations and serial evaluation of family members to determine a final phenotype was possible. However, the family was relatively inbred, the breed in the UK has a relatively small gene pool and it was not certain whether canine microsatellites would show enough polymorphism or sufficient heterozygosity to make this an informative investigation. Consequently, preliminary work was required to investigate whether a linkage analysis study was feasible within this breed.

C.4. *Family studies in Newfoundlands*

C.4.1. *Dogs*

Where there had been a family prevalence of DCM reported by breeders or owners, as many dogs as possible related to affected dogs in several generations were recruited into the study. Most of the dogs in the study population were actually related in an extended family, based on scrutiny of the Kennel Club registration document for each dog.

Each dog recruited into the study had a general history taken and received a full clinical examination prior to a complete echocardiographic / Doppler examination as described previously. In addition, note was made of any relatives known to the owner

of each individual who had been diagnosed with congestive heart failure or other cardiac problems. Such cases were usually included as suspected DCM cases, unless they had been confirmed by echocardiography and / or post mortem examination to have definite DCM, and other heart disease had been excluded.

C.4.2. *Pedigree details*

As well as pet name, owner details and status of each individual, The Kennel Club registered name, date of birth, and names of sire and dam were recorded for each dog. In addition, the numbers of siblings (dogs and bitches) of each dog's litter was recorded and verified whenever possible by consulting either The Kennel Club Breed Record Supplements (Working Group) (KCSB Breed Records Supplements; published quarterly) or the Newfoundland Club records. Details of other Newfoundlands with confirmed DCM which had presented to other veterinary cardiologists were included in the analysis.

C.4.3. *Pedigree analysis and phenotype*

From a copy of the Kennel Club registration pedigree obtained for each dog in the study, with knowledge of the phenotype based on echocardiography and / or post mortem examination, data was input into a pedigree drawing programme, PEDIGREE / DRAW (For the Apple Macintosh, version 4.4; Population Genetics Laboratory, Southwest Foundation for Biomedical Research, San Antonio, Texas), which displayed the extended family tree, as far back as common ancestors, including the kinship coefficient (identical to the coefficient of inbreeding) for each mating, calculated by the software. Males were drawn as squares and females as circles. Dogs known to be deceased were indicated by a diagonal slash, and cause and age of death, if known, was noted. From the family history, confirmed DCM dogs were displayed as filled and suspected DCM (e.g. those without post-mortem or echocardiographic confirmation of the diagnosis, or dogs suffering sudden death) were displayed as hatched shapes. Dogs which had received an echocardiographic examination were indicated the symbol, •. In a similar manner, dogs included in this study identified with clinical and echocardiographic or post mortem abnormalities

conclusively identified as overt or occult DCM were included as solid black shapes and those with equivocal abnormalities were included as shaded shapes (code details given). Dogs who had received one or more echocardiographic examinations were coded as the status of the latest scan. Dogs from which DNA samples had been obtained were also indicated by code ($^{+}$). The Pedigree / Draw (P) number and DNA number specific for each individual included in the study are shown in Appendices B.2. to B.7. from the previous section, in order to locate an individual in both formats. P numbers were also given to common ancestors or important individuals in the family tree as they are important in the pedigree analysis, but details about these dogs are not included. For each dog, a record was made of the number, gender and status of siblings where this information was known or was able to be obtained by the owner or consulting Kennel Club or Newfoundland Club records. Dogs were not classified as confirmed Normal dogs unless they were over eight years old with two successive scans without any echocardiographic or clinical abnormality being identified. Although dogs younger than this without abnormalities at the time of preparation of this thesis were illustrated in the pedigrees as being unaffected, they were included as being of "unknown" status in the later linkage analysis.

The coefficient of inbreeding may be defined as the probability that an individual will have, at a given locus, two genes identical by descent from a common ancestor (Emery 1986a; Nicholas 1987a). The Quaas-Henderson iterative method of computing the kinship coefficient was used by the Pedigree/Draw software (Pedigree/Draw Version 4.4 User's Guide p. 35 (1993); Boyce 1983).

For simplicity of presentation and ease of handling, smaller "nuclear family" type pedigrees were drawn using the PowerPoint (Microsoft) and the Draw facility in Word 7 for Windows (Microsoft), including the coefficient of inbreeding data from Pedigree/Draw. In these family trees, individuals from whom DNA had been obtained are indicated by digits. If no DNA sample had been obtained, then the P number identifying this individual from Pedigree/Draw was used, preceded by P. Dogs not studied but important in the pedigree were indicated by their P number.

The table included in Appendix C.2. indicates the corresponding P and DNA numbers for each individual for dogs included in the study.

Pedigree information was updated after successive echocardiographic evaluations or when information was received from the owner, breeder or the dog's veterinary surgeon.

Where sufficient information was available in a number of generations and siblings in litter, these families were used for statistical analyses in order to hypotheses test for different models of inheritance to attempt to identify the mode of inheritance in these families.

C.4.4. *Description of the Population*

From the number of dogs scanned, the number of males and females in the population were determined. The number of dogs with echocardiographically proven DCM was determined and χ^2 analysis was used to identify any sex predominance.

C.4.5. *Determination of mode of inheritance by observation of pedigrees*

The "nuclear" family trees were scrutinised in an attempt to identify the probable mode of inheritance in this disease, to determine whether data were consistent with a particular mode of simple Mendelian inheritance (Nicholas 1996), the criteria for which are summarised below.

(i) *An autosomal dominant* disorder is transmitted from generation to generation without skipping generations. Every affected animal should have at least one affected parent. Normal offspring do not transmit the disorder to their progeny. Males and females are affected approximately equally. Assuming most matings are heterozygous affected (D/-) to normal (-/-), the segregation frequency between normal and affected should be 0.5. (Mueller & Young, 1995; Nicholas 1996; Strachan & Read 1996c).

(ii) *Autosomal recessive* conditions require common ancestors on both sides of an affected dog's family. Generations may be skipped and affected animals may arise from two normal (but carrier) parents. Males and females are approximately equally affected. In matings between two carriers (d/- and d/-), the segregation frequency of disease is 0.25 (with 0.5 of progeny being carriers and 0.25 genotypically normal, from the Hardy-Weinberg equilibrium). In autosomal recessive conditions, the genetic relationship between parents of affected individuals is greater than parents not producing affected progeny (Mueller & Young, 1995; Nicholas 1996; Strachan & Read 1996c).

(iii) *An X-linked* recessive mode of inheritance would solely or predominantly affect males, or females may be affected to a lesser degree or with older age of onset and transmission of disease should be from mother to son. Generations may be skipped and affected animals may have two normal parents. In an X-linked dominant condition, affected males mated to normal females transmit the disorder to all daughters but no sons. Affected females mated to normal males transmit the disorder to approximately half of their daughters and half their sons. The incidence of the disorder is about twice as common in females than males in the population and every affected offspring has at least one affected parent (Mueller & Young, 1995; Nicholas 1996; Strachan & Read 1996c).

(iv) *Matrilineal inheritance*, from mother to both sons and daughters, would be consistent with a mitochondrial defect resulting in the disease (McMinn & Ross 1995).

C.4.6. *Test for fit for a particular mode of inheritance*

As the condition is believed to be autosomally transmitted, the frequency of the condition in affected families compared with the frequency of the condition in the general population could be compared to test for fit for a particular mode of inheritance. Multifactorial inheritance, i.e. multiple gene effects or a combination of genetic and environmental factors, was also considered. The observed relative frequencies of the disease in siblings (sibs) versus the frequency of the disease in the general population compared with the expected relative frequencies in sibs for various modes of inheritance was tested according to the methodology detailed by Emery (1986c). Expected frequencies are given as 1/2q for an autosomal dominant trait, 1/4q for an autosomal recessive trait and $1/(\sqrt{q})$ for a multifactorial trait, where q is the frequency of the disease in the population.

The frequency of DCM in the general population is not reported in the literature, but during the course of this study (and for the linkage analysis; see later) has been estimated as 0.08 (=q).

A mean frequency of DCM was estimated from the nuclear families, assessing only siblings where at least one case of confirmed DCM had occurred, but including suspect cases and dogs with equivocal echocardiographic abnormalities as being affected. This is the frequency of the disease in sibs (s).

The value of s/q was then compared with the expected frequencies.

C.4.7. *Segregation analysis*

The simplest approach to investigating a mode of inheritance is to compare the observed number of affected individuals in families with the expected number for a particular mode of inheritance (Emery 1986b).

C.4.7.1. *Family 7*

In Family 7 (Appendix C.2.), where there was sufficient data in a litter (as the majority of the litter had been ascertained by echocardiography), statistical analysis was used to identify the ratio of affected to unaffected progeny (at the time of examination) in comparison with the expected ratios for:

(a) an autosomal dominant mode of inheritance
(b) an autosomal recessive mode of inheritance

(since other modes of transmission had been excluded from scrutiny of the pedigree data).

(i) Initially, only confirmed DCM cases were used (suspected but unconfirmed DCM cases or equivocal echocardiographic abnormalities were excluded). Unassessed individuals were treated as being unaffected.

Individuals are identified by their DNA number (excluding the affix NF), where applicable, or their Pedigree/Draw (P) number. Progeny from the following matings were included:

P263 x 43 (2 litters): DCM, 6; Normal, 12 (total 18).
P263 x 7: DCM, 8; Normal, 5 (total 13).

(ii) If suspected DCM cases and cases with equivocal echocardiographic abnormalities were included as affected, this was repeated.

P263 x 43 (2 litters): DCM, 14; Normal, 4 (total 18).
P263 x 7: DCM, 9; Normal, 4 (total 13).

(iii) Unassessed individuals were excluded, and only confirmed DCM cases were assessed.

P263 x 43 (2 litters): DCM, 6; "Normal", 5 (all dFS) (total:11).

P263 x 7: DCM, 8; "Normal", 1 (dFS) (total: 9).

(iv) If equivocal echocardiographic findings were included as affected, but unassessed individuals ignored in the analyses:

P263 x 43 (2 litters): DCM, 11; Normal, 0 (total: 11).

P263 x 7: DCM, 9; Normal, 0 (total: 9)

These scenarios (i), (ii), (iii) and (iv) were tested against the expected ratios for this family under the following hypotheses:

Hypothesis 1.

Dominant mode of inheritance. Bitch 43 normal (-/-). Bitch 7 affected (D/-) and P263 presumed affected (D/-).

P263 x 43 (D/- x -/-): expect segregation ratio normal: affected = 0.5

P263 x 7 (D/- x D/-): expect 0.75 affected (25% homozygously): 0.25 unaffected.

Hypothesis 2.

Dominant mode of inheritance. Bitch 43 is normal (-/-). Bitch 7 is affected D/-. P263 is presumed homozygously affected (D/D).

P263 x 43 (D/D x -/-): expect affected: normal ratio = 1:0

P263 x 7 (D/D x D/-): expect affected: normal ratio = 1:0

Hypothesis 3.

Recessive mode of inheritance. Bitch 43 is normal but a carrier (d/-). Bitch 7 is affected (d/d) and sire P263 is affected (d/d).

P263 x 43 (d/d x d/-): affected: normal ratio = 0.5 (all "normals" are carriers)
P263 x 7 (d/d x d/d): affected: normal ratio = 1:0

Statistical analysis was performed in SigmaStat (v. 2.03 SPSS inc.).

The Chi squared (χ^2) test was employed unless there were insufficient observations in a particular group, where Fisher's Exact test was used instead. Yates correction for continuity was used in the Chi squared (χ^2) test, and the contingency table had one degree of freedom. A significance level of $p<0.05$ was accepted as being statistically significant.

C.4.7.2. *All Nuclear Families.*

From the nuclear families shown in Appendix C.2., the numbers of dogs with confirmed DCM, dogs with confirmed or probable DCM, the numbers of echocardiographically normal dogs (at the time of examination) and the numbers of dogs which were unassessed were determined. Initial analysis concentrated on litters where at least one confirmed case of DCM had occurred. A subsequent analysis included all litters from the nuclear families, to avoid the error of ascertainment.

Statistical comparison used Chi-squared analysis, applying Yates correction and one degree of freedom. Data was compared against (affected: unaffected) ratios of 0.5:0.5 expected for an autosomal dominant mode of inheritance and 0.25:0.75 for an autosomal recessive mode of inheritance. Probable (suspected cases or cases with equivocal echocardiographic abnormalities) and confirmed DCM cases were grouped together, and unaffected (at the time of examination) and unexamined individuals were also grouped.

C.4.7.2.1. *The Proband Method of Segregation Analysis*

This method of segregation analysis is sometimes referred to as Weinberg's "proband" method and is detailed by Emery (1986b). It takes into account that there

may be more than one proband in each litter, but not all affected dogs are probands. The simplest method of determining the proportion of affected sibs to probands is to count each sibship twice for each time it has been independently ascertained, omitting the proband each time.

$$p = \frac{\text{Affected sibs}}{\text{Total sibs}}$$

This analysis was applied to the nuclear families, including litters where at least one confirmed case of DCM was documented, although suspect cases and dogs with equivocal echocardiographic abnormalities were included in the analysis as affected sibs. This result was compared against the expected ratio of affected: normal progeny under an autosomal dominant and an autosomal recessive hypotheses models, using χ^2 analysis (SigmaStat (v.2.03; SPSS inc.)).

C.4.7.2.2. *The Singles Method of Segregation Analysis.*

The Singles method is the simplest, quickest and most efficient form of segregation analysis and is possible without the aid of computer software (Emery 1986b; Nicholas 1987b). Normally, all members of each reported family must be included in the data. One of two assumptions have to be made:

(i) either all families with affected offspring are included
(ii) a random sample of families with affected offspring have been included (Nicholas 1987b).

The latter assumption may be made for the nuclear family data presented here.

The segregation frequency may be estimated as described by Nicholas (1987b) as:

$$\text{"p"} = \frac{A - A_1}{T - A_1}$$

and its estimated variance ("p_{var}") is approximately:

$$(\text{“}p_{var}\text{”}) = \frac{(T - A)}{(T - A_1)^3} \times \left\{ A - A_1 + 2A_2 \frac{(T - A)}{(T - A_1)} \right\}$$

(*Abbreviations*: A = total number of affected offspring in the available data, T = total number of offspring in available data, A_1 is the total number of families with just one affected offspring and A_2 is the total number of families with two affected offspring. (Nicholas 1987b)).

The "p" result and its variance ("p_{var}") is compared with the "null hypothesis" value of p_0 of 0.25 for an autosomal recessive mode of inheritance and 0.5 for an autosomal dominant mode of inheritance (Nicholas 1987b). The test statistic is given by:

$$Z^2 = (\text{“}p\text{”} - p_0)^2 / (\text{“}p_{var}\text{”})$$

Values of Z^2 are distributed approximately as χ^2 and were therefore compared with data given on a Chi squared distribution table, for one degree of freedom (Table B5. p.523. In Practical Statistics for Medical Research. D.G. Altman (1991). Chapman & Hall. London.)

The nuclear family data was used. The initial analysis included only dogs with confirmed DCM, and the total analysis included probable DCM dogs. Values for "p" and its variance ("p_{var}") were calculated with a scientific calculator and the null hypothesis was tested for autosomal recessive and autosomal dominant modes of inheritance.

C.5. *Obtaining DNA samples*

C.5.1. *Blood sampling*

Dogs which were from the extended family had a blood sample obtained for a renal health check and DNA storage, with owner consent. The renal health check, assessing serum urea and creatinine, was believed to be useful, particularly in older animals or in animals identified with cardiac disease prior to treatment intervention, but also in all Newfoundlands as owners were increasingly concerned both about Cysteinuria and Glomerulonephropathy in this breed (Booth, 1990; Koeman *et al* 1994; Casal *et al* 1995).

The hair coat was clipped over the jugular furrow. Hibitane / surgical spirit mix was sprayed onto the hair coat in attempt at asepsis. A blood sample of approximately seven millilitres was obtained by jugular venipuncture with a 20 gauge one inch needle (Microlance^{R}) and divided into one tube without anticoagulant (Vacutainer, plain Becton & Dickinson) (2 mls) and one tube with Potassium EDTA as the anticoagulent (Vacutainer, EDTA K_3; Becton & Dickinson) (5 mls). The tubes with clotted blood were submitted to the Clinical Laboratory, Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies, University of Edinburgh for assessment of renal function (serum urea and creatinine concentrations). The EDTA anticoagulated blood was used for extraction of genomic DNA. Samples were stored in a refrigerator until return to Edinburgh. Although samples were submitted as soon as possible for laboratory analysis or for DNA extraction, there was inevitably a significant time delay for some samples taken early during a scanning trip.

C.5.2. *Genomic DNA extraction*

A genomic DNA extraction kit was used (Nucleon^{R} BACC2, Nucleon Biosciences, Scotlab Bioscience, Coatbridge, Scotland), according to the manufacturers instructions unless otherwise stated. Each five millilitre sample was decanted into 50 ml polypropylene tubes with conical bottom (Nunc) with ten millilitres of Reagent A. Reagent A consists of 10 mM Tris-HCl, 320mM sucrose, 5mM $MgCl_2$, 1% Triton X-100 and 40% NaOH added to adjust reagent to pH 8.0. The purpose of Reagent A was to lyse the red blood cells (RBCs) leaving white blood cells (WBCs) (containing genomic DNA) in tact. Reagent A and the blood were mixed by placing onto a roller (Roller SRT1; Stuart Scientific) for four minutes at room temperature. The samples were then centrifuged at room temperature in a Beckman GPR Centrifuge, which was 30 cm diameter, for 4 minutes. Kit instructions stated the force of centrifugation should be 1300g (corresponding to 1800 rpm in this centrifuge), but during extraction procedures, it was discovered that 3000 rpm more consistently resulted in a stable WBC pellet. At lower centrifugation, sometimes the cell pellet was easily lost into the haemolysed RBC supernattent during sample handling. Leaving the cell pellet

undisturbed in the bottom of the conical tubes, the supernattent was aspirated and discarded. Two millilitres of Reagent B were then added. Reagent B consists of 400mM Tris-HCl, 60mM EDTA, 150mM NaCl, 1% SDS and 40% NaOH added to to adjust the pH to 8.0. Reagent B is used to lyse the WBCs. The pellet of WBCs and reagent B were vortexed in a WhirliMixer (FSA Laboratory Supplies). The instructions stated that brief vortex was sufficient but it was found that this did not resuspend the cell pellet; vortex over a longer period was required. It was hoped that this did not lead to fragmentation of the DNA. If the sample was not adequately resuspended, this led to later difficulties in separation of DNA and protein containing layers. Following resuspension of the lysed cell pellet in reagent B, the entire contents of the tube were decanted into a 5ml polypropylene tube with maximum internal diameter of 12 mm. 500µl Sodium Perchlorate were added for deproteinisation of the sample and the reagents mixed by repeated gentle inversion (seven times). Two millilitres of chloroform were added to the mixture, to aid in the separation of the DNA and protein containing layers. This was mixed by gentle repeated inversions to initially emulsify the phases. The contents of the tube were centrifuged for 1 minute at 800g (corresponding to 1400 rpm of the 30 cm diameter centrifuge). This led to separation of protein in chloroform at the bottom, a layer of cell debris at the interface and an aqueous layer containing DNA at the top. 300µl of Nucleon^{R} resin were added into the tube and without re-mixing, the sample was recentrifuged at 1300g (corresponding to <1800 rpm) for 3 minutes. This resulted in the resin sitting over the layer of cellular debris and the lower organic phase, so separating the DNA containing upper aqueous phase. If the resuspension of the cell pellet in reagent B had been inadequate, it was found that a mucoid cell plug tracked across this resin layer from the debris layer into the aqueous DNA containing layer and remained adherent to the resulting DNA plug, so contaminating it.

The upper aqueous DNA containing layer was aspirated by pipette without disturbing the Nucleon^{R} resin layer into fresh polypropylene tubes. The sample was re-centrifuged briefly to ensure any resin contamination of the sample was pelleted, and the upper layer was transferred into another clean polypropylene tube. To precipitate

the DNA, twice the volume of absolute (100%) ethanol was added to the volume of the sample. Gentle inversion allowed visible DNA precipitation which formed a white cotton wool like mat in the tube. The DNA was then pelleted by centrifugation at 4000 g (corresponding to 3400 rpm) for 5 minutes. The alcohol supernattent was aspirated by pipette and discarded. The DNA pellet was washed, by adding 2 ml 70% ethanol and mixing on the rotary mixer for about 5 - 10 minutes. The pellet was recentrifuged and the alcohol aspirated and discarded. The tubes with the DNA pellet were left open to allow the sample to dry at room temperature for about 15 minutes. The DNA sample was resuspended in 1 ml Tris EDTA (made up from 100 µl 1M Tris, 20 µl 0.5M EDTA and 9.88 mls distilled H_2O) and allowed to dissolve by rotary mixing over 2 hours or until dissolution was complete. The samples were then transferred by pipette into 1.8 ml cryo tubes (Nunc), the tubes were labelled by permanent marker and the samples stored at -80°C for prospective molecular genetic analysis.

C.6. *Determination of quantity and quality of Genomic DNA stored*

C.6.1. *Quantification*

The Newfoundland DNA samples were labelled consecutively based on the order in which they had been obtained, and ranged from NF001 to NF090 at the time this work was done. The genomic DNA samples suspended in Tris EDTA (TE) were transferred from the freezer to a refrigerator to allow them to thaw. Using a spectrophotometric technique (Sambrook *et al* 1989), the quantity of DNA was determined. Briefly, spectrophotometry at 260 and 280 nanometres (nm) is recorded. The optical density (OD) at 260 nm allows determination of the amount of nucleic acid in the sample, using specific coefficients, depending on the type of nucleic acid present in the sample. An OD of 1.0 represents 50 µl/ml of double stranded DNA (e.g. genomic DNA), or 40 microlitres per millilitre (µl/ml) of single stranded DNA or RNA, or 20 µl/ml of single stranded oligonucleotides. Pure DNA should have a ratio of 1.8:1 for the OD at 260nm:280 nm. If samples are significantly contaminated

with protein (or phenol; not used in the Nucleon extraction kit), the ratio is significantly less than this.

As the samples are suspended in 1 ml of TE, concentrations were determined as micrograms/millilitre (μg/ml). Fifty μl of the sample was added to 700 μl distilled water (dH_2O), giving a dilution of fifteen fold. The concentration of DNA was then determined:

$$\text{conc. DNA} = \text{Dilution (15)} \times \text{Coefficient (50 mg/ml)} \times OD_{260}$$

The units are μg/ml. Conventionally, DNA concentrations are given as μg/ μl, so this result was divided by 1000.

For the diluted samples, the ODs at 260 nm and 280 nm were recorded, and the ratio of these was calculated for each sample.

C.6.2. *To check structure of genomic DNA*

As another check on the quality of the DNA and to ensure that the extraction procedure had not resulted in significant fragmentation of the genomic samples, a 0.8% agarose gel was prepared, using 0.8 g agarose in 100mls TBE (Tris-Boric acid-Ethylenediamino-tetraceacetate at pH 8.0), by microwaving the ingredients in a flask until boiling to dissolve the agarose, and then allowing the contents to cool to hand-warm, before incorporating ethidium bromide (EtBr) (one microlitre) prior to pouring the gels. EtBr intercalates between base pairs, allowing migration of DNA to be visualised. Agarose gels incorporating EtBr staining were visualised using ultraviolet transillumination in a dark room and photographed by thermal printer. Five microlitres of the size marker λ *hind III* was placed in the left hand well of each lane. Placed in each of the other wells was one microlitre of genomic DNA mixed with nine microlitres of Orange G. Once loaded, the gels were placed into TBE buffer in an electrophoresis gel tank and allowed to run at 80 Volts for two hours until the initial three bands of λ *hind III* were separated. These three bands correspond to

sizes in base pairs (bp) and DNA quantity (nanograms, ng) of: 23130 bp; 120 ng, 9416 bp; 48.5 ng and 6557 bp; 33.8 ng. Comparison of the migration distance and the band intensity allowed the approximate size of the genomic DNA and the quantity of DNA from the band intensity to be estimated from these gels.

C.7. *Identification of a subgroup of the Newfoundland extended family informative for genetic linkage analysis.*

Clinical and echocardiographic data to phenotype individuals correct at the time of this pilot study were utilised. Dogs closely related were selected, with at least two individuals in a generation where possible, to allow determination of the phase and where DNA had been obtained from as many individuals as possible. As it was unfortunately rare that DNA had been obtained from both parents, in some cases a number of progeny in each generation were selected which, if there was sufficient polymorphism and heterozygosity of microsatellites among the dogs, may indicate the microsatellite genotype data of the missing parent.

Dogs incorporated into this study are displayed in Figures C.1.a., C.1.b. and C.1.c. (Volume II).

A simulated linkage program (Simlink; Ploughman & Boehnke 1989; Terwilliger & Ott 1994a) was run to determine whether the selected dogs were in a family group sufficiently informative for a genetic linkage analysis study. Data was input in standard linkage format (Terwilliger & Ott 1994), with dogs with equivocal echocardiographic abnormalities either included as "unknown" or assigned as affected. An autosomal dominant mode of inheritance with complete penetrance was assumed. Disease prevalence was estimated as 8%, giving a disease allele frequency of 0.04.

Individuals from this family were selected once protocols for determining genotyping data were resolved. These were (DNA identity, NF-): 80, 81, 9, 84, 75, 76, 89, 14, 83, 82, 43, 7, 59, 56, 70, 49, 54, 45, 53, 58, 63, 86, 69, 42, 8, 31, 33, 4, 20, 5, 26, 32,

28, 30, 22, 41 and 6, with a negative control. As 40 lanes per ALF gel (see later) were available and lanes 1 and 40 are left empty to prevent artefact, these individuals comprised one ALF run.

C.8. *Amplification of canine microsatellites by the polymerase chain reaction (PCR)*

Canine microsatellite primers were obtained from the Animal Health Trust, Newmarket. As well as a reference name or number for the microsatellite, the concentrations of the forward and reverse primers and the PCR conditions required for each primer set (viz. annealing temperature, magnesium concentration etc.) are displayed in Table C.2.a The expected product sizes and the polymorphism information content (PIC) where known were also given. Some of the microsatellites have been previously or subsequently published, and this information is also given in Table C.2.a. (Volume II).

The forward and reverse primers were prepared if necessary to adjust them to concentration of 50 ng/µl, by diluting with sterile dH_2O. A mixture of dNTPs (2'-deoxynucleoside 5'-triphosphate) was prepared using each of the set (100 mM concentration; Advanced Biotechnologies Ltd.). To make a 10 mM concentration of dNTPs, 40 µl of each of dATP (deoxyadenosine triphosphate), dCTP (deoxycytidine triphosphate), dGTP (deoxyguanosine triphospate) and dTTP (deoxythymidine triphosphate) were added to 240 µl dH_2O and retained as a stock solution.

All ingredients and samples were prepared and stored in an ice bath. Ingredients were set up for 25 µl (or 10 µl) reactions. Incorporated into each reaction was dH_2O, the amount calculated from the difference between 25 µl (10 µl) and the total volume of the rest of the ingredients, Buffer (10 x PCR Buffer; Perkin Elmer): 2.5 µl (1 µl); dNTPs from stock dilution; 0.5 µl (0.2 µl) and Magnesium chloride ($MgCl_2$ solution, 25mM; Perkin Elmer), the quantity depending on the required conditions for each primer set, so 2.0 µl (0.8 µl) for a 2mM [Mg^{++}] or 1.0 µl (0.4 µl) for a 1mM [Mg^{++}].

Into each reaction. 2.0 µl (0.8 µl) of each primer (forward and reverse), 0.2 µl (0.08 µl) *Taq* DNA polymerase (Amplitaq DNA polymerase; Perkin Elmer) and 1 µl (0.4 µl) of genomic DNA was added. Separate Gilson^{R} pipettes were used for setting up the PCR reactions and for adding the genomic DNA.

Once the individual reactions were set up in small ependorf tubes, they were covered with two drops (Pasteur pipette) of mineral oil to prevent evaporation, and loaded into a thermal cycler block (Hybaid Omnigene). This was programmed depending on the primer conditions required. For normal PCR, an initial denaturing phase was run, for seven minutes at 94°C (to allow separation of the double stranded DNA strands). Thirty five cycles of the following phases were then run: 94°C for 45 seconds, 55°C (or primer specific annealing temperature) for 45 seconds and then 72°C for 45 seconds. Finally, a ten minute phase at 72°C completed the reaction. During the cyclical phase, the 94°C temperature is required to separate DNA strands, the annealing temperature (e.g. 55°C) is required for primers to anneal to complimentary strands of DNA, and then 72°C is the optimal temperature for the action of *Taq* (*Thermus aquaticus*) DNA polymerase which results in exponential increase in the amount of product specific to the primers. The final 72°C phase allows all the DNA to become double stranded again.

For each PCR run, a negative control was always run (with sterile dH_2O instead of genomic DNA) to check for DNA contamination of reagents.

C.8.1. *Taq Gold*

The Animal Health Trust routinely uses Amplitaq Gold (personal communication, M. Binns). Amplitaq Gold (Perkin Elmer) will only function at high temperatures and so this avoids spurious mis-priming resulting in additional non-specific bands or peaks during preparation of samples prior to starting the thermal cycling. Indeed, it is said to obviate the need to prepare samples on ice, although this was not carried out in this pilot study; sample preparation was similar for both standard *Taq* and *Taq* Gold. Routinely, standard *Taq* is used by the MRC Human Genetics Unit and so an

experiment was performed to assess whether use of *Taq* Gold did result in significant improvement of product specificity and reduction in mispriming events. For primer pairs E41, A15, A29, A19 and F38, all of which had shown less than optimal results with standard *Taq*, PCR reactions were set up using three DNA samples for each primer set (NF071, NF072 and NF073). Product detection was on a Nuseive EtBr gel using ultraviolet transillumination (see later).

C.8.2. *Touchdown PCR*

Some of the primer conditions reported by the Animal Health Trust as "Touchdown" PCR or TD PCR, with the final annealing temperature given (e.g. Holmes *et al* 1994). This was another solution to circumvent the problem of spurious bands or peaks due to mis-priming. Mis-priming events may dominate the PCR reaction and are more severe if the target DNA template is only present in small amounts. Increasing the annealing temperature and / or adjusting the $[Mg^{++}]$ concentration may improve the specificity of the amplification provided that the spurious interactions are significantly less stable than the specific ones (Don *et al* 1991). Don and co-workers (1991) described a method of taking advantage of the exponential nature of PCR reactions by starting 10°C above the annealing temperature and reducing the temperature by 1°C every second cycle to "touchdown" at the final annealing temperature. Any difference between correct and incorrect annealings for a given temperature will give an advantage to amplification of the correct template sequence.

When TD PCR was indicated, because there were an insufficient number of programming steps offered by the PCR machine to absolutely follow the protocol of Don and colleagues (1991), this was modified. The thermal cycler was programmed with initial annealing temperature 10°C above the final temperature, with reduction of 2°C every four cycles until 2°C above the final temperature, then the temperature was reduced by 1°C for two cycles before finally arriving at the annealing temperature, which was maintained for another fifteen cycles.

To assess whether TD PCR was a necessary step or to indicate whether the use of *Taq* Gold obviated the requirement for cumbersome TD procedures, primer pairs K200, K209, K212, K39, K32, K120 and K292 each with DNA samples from NF071, NF072, NF073, NF074 and NF090 were set up in reactions either by using Amplitaq Gold or by using the TD PCR technique. The products were read in a Nusieve EtBr gel using ultraviolet transillumination (see later).

C.9. *Fluorescent dUTPs to label PCR products*

C.9.1. *Experiment to assess fluorescent dUTPs for labelling PCR products*

Some of the primers were supplied unlabelled with laser fluorescent dye. When product visualisation using the ALF was carried out (see later) a technique for fluorescently labelling products during the PCR reaction itself had to be developed. Normally, fluorescein is used during primer synthesis to label one primer strand for detection by the ALF system. Unfortunately, after consultation with Pharmacia, it was determined that labelling products during the PCR reaction would be prohibitively expensive, had not been carried out previously to the company's knowledge and may not result in successful ALF detection of products.

However, the use of fluorescent dUTPs has been recently described to label products during the PCR reaction, for detection by the ABI systems (Applied Biosystems, Perkin Elmer) (Rhodes *et al* 1997). Three dyes are available from Perkin Elmer: R110 (blue), R6G (green) and TAMRA (yellow). Simple addition of the FdUTPs rather than alteration of the optimised dNTP concentrations was reported to provide sufficient incorporation into product to allow reliable detection by the ABIs (Rhodes *et al* 1997).

As it was uncertain which FdUTP would be optimally detected by the ALF, a decision was made in part on the basis of cost. The Animal Health Trust normally used R110 (personal communication, M. Binns). The desired concentration of [R110]dUTP is reported as 0.5 μM (Rhodes *et al* 1997) which was calculated to

correspond to five picomoles per ten microlitre reaction. [R110]dUTP contains three nanomoles in 30 microlitres (concentration 100μM). One microlitre was diluted with 19 μl 1xPCR buffer to enable the addition of one microlitre of this dilution into the PCR reaction to produce the correct concentration. [R6G]dUTP is reported to be incorporated into PCR reactions at a concentration of 1 μM (Rhodes *et al* 1997). It is also available as 3 nmoles/ 30μl at concentration of 100μM. One microlitre was diluted in 9 μl 1xPCR buffer so that the addition of one microlitre of this diluted mixture gave optimal concentration in the PCR reaction. The final concentration of TAMRA in a PCR reaction is reported to be 8 μM (Rhodes *et al* 1997) which meant that each 10 μl PCR reaction would cost 90p and the overall cost was found to be prohibitive.

Both [R110]dUTP and [R6G]dUTP were assessed in PCR reactions in the ALF system. Using one primer pair (A15) and five DNA samples (NF071, NF072, NF073, NF074 and NF090), it was found that R110 resulted in peaks of greater amplitude and so this and the cheaper cost meant that it was subsequently used for fluorescently labelling PCR reactions (see Results).

C.9.2. *Improvement of product detection: Use of T4 DNA Polymerase*

The use of FdUTPs incorporated in the PCR reaction results in broader peaks, and for dinucleotide repeat sequences where two alleles are just two base pairs apart, accurate genotyping may not be possible. In part, this problem is due to the tendency of *Taq* polymerase to add an extra base at the 3' end of the amplified fragments in template independent manner (Ginot *et al* 1996). This results in a detectable peak one base mobility unit larger than the actual allele base pair size, which, with broad, poorly defined peaks, may be difficult to identify. One solution to this problem reported by Ginot and colleagues (1996) was to correct for the one base overhangs by the enzymatic removal of such bases by treating with T4 DNA Polymerase after the PCR reaction.

To assess whether this procedure did improve genotype results, as part of the experiment to assess the FdUTPs R110 and R6G, using primer pair A15 and DNA samples NF071, NF072, NF073, NF074 and NF090, for each FdUTP assessed, products from the PCR reaction were either not treated or were treated with T4 DNA Polymerase.

Initially, once the PCR was complete, as much mineral oil as possible was removed by pipette from the layer covering the products. T4 DNA Polymerase (1 unit/μl) and T4 DNA Polymerase Buffer (x5) (Boehringer Mannheim) were used and 0.1 μl (0.1 unit) of T4 DNA polymerase was added to 2 μl of 5X T4 DNA Polymerase buffer for each sample destined to receive this treatment (although made up initially as a master mix and then 2.1 μl of the mixture was added to each 10 μl PCR reaction after completion of thermal cycling). The samples receiving the treatment were then placed in a water bath for thirty minutes at 37°C.

As the results (see later) showed that for this primer set and these DNA samples, there was no major difference detected by the T4 DNA polymerase treatment, this step was not routinely used.

C.9.3. *Phenol/Chloroform Extraction*

The use of FdUTPs to label the PCR reactions inevitably resulted in non-incorporation of some of the FdUTP. As the FdUTP size is approximately 200 bp, it was judged to be important to "clean up" the DNA of the product to prevent co-migration of the FdUTPs from confounding detection and genotyping of the alleles.

Methods detailed by the Animal Health Trust (personal communication, M. Binns) were used. A master mix containing equal volumes of phenol (buffer saturated; Gibco-BRL) and chloroform isoamyl alcohol was made up. An equal volume of phenol / chloroform was added to each sample (12.1 μl to samples treated with T4 polymerase and 10 μl to samples not so treated). Samples were then vortexed briefly and then stored on ice for five minutes. After this, each sample was centrifuged at

14000 rpm for five minutes. The upper aqueous layer containing DNA was removed by pipette (through the upper residual mineral oil) and placed in a fresh labelled ependorf tube. The samples were then prepared and assessed by ALF (see later).

C.10. *Determining PCR products*

At the end of the PCR, the success of the reaction was assessed.

C.10.1. *Agarose gel with Ethidium Bromide (EtBr)*

In the EtBr technique, 5 µl Orange G was added below the mineral oil layer, or was mixed with 12 µl PCR product on Parafilm^{R}, depending if the remains of the product was to be used in another gel. To assess whether PCR products were obtained and to assess whether an agarose gel would be suitable for differentiating between product sizes, some of the products were run by electrophoresis on a 4% Nusieve agarose gel with EtBr, with band detection by ultraviolet transillumination. The size marker ϕX174 *Hae*III was run in the left-most wells to attempt to quantify product sizes, by noting the distance each band migrated and plotting a size curve on log-linear graph paper. From the migration distance from the well for each product, an estimate of its base pair size could then be determined. Product detection using the Nusieve gel with EtBr was assessed initially for primer set PEZ18 which had resulted in bright product bands on the agarose gel, and all 90 DNA samples were assessed in order to determine the ease of allele size differentiation.

C.10.2. *A.L.F.*

Some of the primers supplied by the Animal Health Trust had the forward strand fluorescently labelled with HEX (green), FAM (Blue) or TAMRA (yellow) (Perkin Elmer) for use on the ABI377 (Applied Biosystems, Perkin Elmer). Normally, the laser fluorescence system, (Pharmacia LKB Automated Laser Fluorescence (A.L.F.) Sequencer; Pharmacia Biotech) uses forward primer labelled with fluorescein during primer synthesis (Mansfield *et al* 1994). However, the ABI dyes were shown to result in detectable peaks on this system.

The ALF system uses polyacrylamide gel electrophoresis (PAGE) with detection of laser fluorescence by individual photodiodes for each lane of the gel (Mansfield *et al* 1994). After thorough cleaning of the glass plates, prior to constructing the plates, to ensure the top of the gel was secure prior to removing the comb, both plates had the top one centimetre coated with bindsaline with acetic acid. The plates were separated with spacers and the laser transmission glass and locked together.

Gels had been pre-prepared, aliquotted in 60 ml syringes and stored in a refrigerator. The gel mixture consisted of 840 g urea, 240 mls Lone Ranger^{R} gel, 120 mls 10x TBE, made up to two liters with dH_2O. 10xTBE was made up using 242.28 g Tris, 102.64 g boric acid and 7.44 g EDTA, made up to two litres with dH_2O.

The gel mixture was decanted into a mixing pot and 100 µl APS (Ammonium persulphate 10% solution) and 52.5 µl Temed was added to it, mixed, and the contents aspirated back into the syringe. The gel was then poured into the horizontal plates, spreading by capillary attraction, ensuring no air bubbles were trapped (if introduced, they were fished out with an acetate hook). The comb was inserted into the top of the gel, clamped with bulldog clamps, and the gel allowed to set for a minimum of two hours.

For some gel runs, a novel gel under assessment by the MRC Human Genetics Unit (UV Gel 500, Pharmacia) using ultraviolet polymerisation was used. These gels set quickly but ran more slowly on the ALF.

Once set, the comb was gently removed and the plate fitted onto the ALF machine, into a bottom buffer tank and, after securing, the top and bottom buffer tanks were filled with 0.6 TBE (1.0 TBE for the UV gel). Each gel could be routinely used for two runs. The computer started the gel and was set at maximal voltage (2000V and maximum current (70 mA) at a temperature of 50°C, with laser power of 3 mW. Sampling interval was every 1.75 seconds and the running time usually about 140

minutes. The laser beam alignment was checked and corrected if necessary, and the water pre-heat to control plate temperature was set.

The ALF dye for assisting the loading of the wells was a mixture of dextran blue, formaldehyde and EDTA. Size markers, one greater than and one less than the expected product size range, were added. From a stock mixture of 2:1:1 of dye:small:large size marker, four microlitres was pipetted into marked wells on a labelled plate. Into each well, 0.5 μl each PCR product was added. The plate was heated for two minutes at 94°C to denature and separate the double stranded DNA. The gel wells were then loaded with the contents of the plate wells and a note made of which lane each sample was loaded. The plates were marked into ten "clones" numbered one to ten, each separated into four lanes labelled (left to right) A,C,T and, G. The run was then initiated. The computer screen could display one clone in turn, with each lane (A, C, T or G) colour coded as green, blue, yellow and red. Once PAGE resulted in either a size marker or a product to pass through the laser beam, this was displayed as a peak in the appropriate colour.

Once the run was complete, the run was saved and given a file name. The results were printed out for each clone, by selecting the appropriate region between two size markers. The time scale was printed at the bottom of each page.

The data was copied in Telnet to a PC. Analysis used the ALP (Automated Linkage Preprocessor) program. Initially, the gel was checked using the Fragment Manager conversion program (Pharmacia) to detect and tabulate the fluorescence peaks for each lane (Mansfield *et al* 1994). Aligning the size markers in each lane, these peaks were checked to ensure that they all fell within $\pm$ 1 minute i.e. that the gel had run uniformly across its width. In the ALP program, the known small and large size marker size in base pairs was input as standard 1 and standard 3 respectively, and lane 20 was normally used (the centre of the gel) as the standard lane. A note was made from the fragment manager the modal time each standard peak arose and the time intervals between which all the product peaks fell. Where there was a known

repeat size for the microsatellite being amplified (e.g. four for a tetranucleotide repeat or two for a dinucleotide repeat), this was included. Stutter was set at 70% (so peaks preceding the main peak <70% of the amplitude of the main peak were ignored by the program). The locus was recorded as the primer identity. Once all the required data was input, the analysis was run.

From the report, the size of peaks (as base pair mobility units) was recorded with the intensity (amplitude) of each peak. This was checked against the ALF printouts, particularly in a noisy gel or a gel with subsidiary peaks due to mis-priming, stutter etc. For each primer pair, the alleles as base pair sizes for individual dog were recorded.

C.11. *Microsatellite genotyping data from the Newfoundland sub-family generated by the ALF/ALP system*

Acquisition of the genotyping data may best be described as semi-automated, since it depended on visualisation of peaks from the ALF printouts, recognition of any stutter or other spurious bands and the genotyping data generated by ALP. In the case where some products were re-run or PCR reactions repeated, one or more individuals products with well defined alleles with size determined by this system were retained (frozen at -20°C until required) and used as "gold standards". As different gels were used and mobility may in any case vary between runs, this was important in order to obtain comparable data. For printout of ALF data from each ALF run, the scale was normally maintained, displaying the product peaks between the large and small size markers, so that comparison of clones to compare product size and appearance was possible by superimposition of the size markers.

Genotyping data was obtained for all 90 dogs for the microsatellite PEZ9 (data not shown). Subsequently, for the other primer pairs, the sub-family of 38 individuals, based on the pedigrees illustrated in Figures C.1.a., C.1.b. and C.1.c. used in the simulated linkage analysis study, were used. These were dogs (NF-) 80, 81, 9, 84, 75, 76, 83, 14, 83, 82, 43, 7, 59, 56, 70, 49, 54, 45, 53, 58, 63, 86, 69, 42, 8, 38, 31, 33, 4,

20, 5, 26, 32, 28, 30, 22, 41 and 6. The use of 38 individuals meant that each primer pair occupied one ALF gel (40 lanes, leaving lane empty and lane 40 with the negative control (no DNA)). These genotyping data were drawn under the appropriate individual in the pedigrees shown as Figures C.1.a., C.1.b. and C.1.c. This was in order to confirm normal Mendelian inheritance was followed for each microsatellite. If Mendelian inheritance could not be identified, then the primer pair was discarded as being not useful for a linkage study in this family of dogs.

If some data were repeated (for example, due to a failed PCR reaction or an error in lane loading, resulting in no data in a particular well), the PCR products of at least two individuals with different allele sizes were retained as "gold standards" in order to compare new data with previous gels.

For some of the complex genotype appearance produced by some primer pairs, particularly where FdUTPs had been used, Dr. Nigel Holmes and Ed Ryder at the Animal Health Trust were consulted about allele calling. A general principle was maintained: alleles must retain the same appearance, however complex. Allele calling based on similar appearance was therefore important. In addition, scrutiny of all the family data should allow recognition of homozygotes and heterozygotes. For complex genotype appearance, matching these with the pedigrees and assuming Mendelian inheritance, sometimes assisted in allele calling.

From the pedigree, if a marker showed sufficient heterozygosity and polymorphism, it was sometimes possible to infer a genotype for a missing parent, from the genotypes of progeny and known parent.

C.12. *Linkage Analysis*

The linkage analysis software available at the Human Genome Mapping Project Resource Centre (HGMP-RC) at Hinxton, Cambridge was used accessed via the World Wide Web (Netscape) (address: http://www.hgmp.mrc.ac.uk). These programmes are Unix based. The FASTLINK programs were used two-point linkage

analysis (Lathrop & Lalouel 1984; Lathrop *et al* 1984; Cottingham *et al* 1993; Schaffer *et al* 1994) particularly MLINK.

A dominant mode of inheritance was presumed. The disease prevalence was estimated to by 8% in a population (see previously). If the D allele is responsible for DCM, and - corresponds to "wild type", then the population may be divided into D/D, D/- and -/-, where disease may be associated with the D/D homozygotes or the D/- heterozygotes. This gives an allele frequency of 4% in the population, with 96% of the population carrying the wild type allele (0.04: 0.96). The disease was initially considered to be 100% penetrant in dogs over eight years old; in younger dogs, status was included as "unknown" unless they had equivocal echocardiographic abnormality suggesting that they were affected.

As microsatellite marker allele frequencies are unpublished in the Newfoundland population, allele frequencies were calculated from the genotyping data generated by the Newfoundland sub-family group. Rather than base pair size (Tables C.3.a. and C.3.b.), the alleles were re-labelled from the smallest to the largest as allele one upwards. The genotyping data for each dog could then be tabulated or imported into the linkage programs as a simple integer. Data used in the linkage analysis are displayed in Tables C.4. and C.5.

Input into the linkage programs was as described in Terwilleger and Ott 1994(a-d). For linkage format, the individual identity has to be numbered from one upwards, sequentially. Consequently, the individuals on the pedigree were renumbered sequentially, as shown in Figures C.2.a., C.2.b. & C.2.c. The pedigree data illustrated in Figures C.1.b. and C.1.c. were input in linkage format, together with the common ancestry detailed at the top of Figure C.1.a. The number of extra loops in the pedigree without adding significantly to the informativeness resulted in the rest of the pedigree data from Figure C.1.a. from being excluded. Input into the pedigree file included pedigree identity, individual numerical identity, the individual's father's identity and mother's identity in the next two columns (0 0 if a founder), the sex of

the individual was indicated as 1 (male) or 2 (female). In the next column, the affectation status was input as 1 (unaffected), 2 (DCM) or 0 (unknown). Dogs which were echocardiographically normal but were less than eight years old were classified as "unknowns". In columns seven and eight, the genotype data (as allele numbers) was input for each microsatellite.

For the pedigree input, the input was based on the file written in PICO, and was similar to the following, and saved with extension -.pre. The input for the data for PEZ11 is shown in Table C.6 as an example.

Because there were a number of loops present in this pedigree, these had to be broken to allow the analysis to proceed. The maximal number of loops had to be reset to allow 20 loops in order to allow the analysis to be run. Loop breakers were selected which were parents with progeny and preferably genotypically informative, based on the advice in Terwilliger and Ott (1994e). The loop breakers selected are indicated by encircling in Figures C.2.a., C.2.b. & C.2.c. The loops.out file formed after the command MAKEPED was run detailed the presence of loops identifying offending individuals which helped in making this selection. After MAKEPED was run, a new file with extension -.ped was created. The PREPLINK command was used to generate a parameter file. Two loci (the affection and marker locus) were identified. The dominant inheritance and complete penetrance was included, with disease allele frequency set at 0.04 (wild-type 0.96). The number of alleles for the microsatellite marker locus with the allele frequencies which had been calculated for each allele (Table C.4.) were included for each marker. Data was input as indicated in Terwilliger & Ott (1994b). Once the parameter file details were complete, it was saved as a -.dat file. Two point linkage analysis (MLINK) was run using the linkage control program, LCP (Terwilliger & Ott 1994d). Recombination increment was set at 0.1, to allow LOD scores to be calculated at 0, 0.1, 0.2, 0.3, 0.4 and 0.5. The -.ped and -.dat files with the corresponding genotype data were included and PEDIN was run, which calculated LOD scores at given values of theta.

PRELIMINARY WORK TO INVESTIGATE THE POTENTIAL FOR GENETIC LINKAGE ANALYSIS IN NEWFOUNDLAND FAMILIAL DCM

RESULTS

C.13. *Population description*

The general population was considered as all dogs scanned. One hundred and sixty five individuals were scanned, 69 males and 96 females. There were twelve male and seventeen female cases of DCM identified in this population. Chi squared analysis showed no significant gender difference in DCM when compared with the total scanned population.

The prevalence of DCM in the scanned population was 17.6%, although it was known that there was a bias in that families presented with a known prevalence for DCM. Consequently, arbitrarily, a disease prevalence was estimated as 8% in the linkage analysis (see later).

C.14. *Determination of mode of inheritance by observation of pedigrees*

Full pedigrees using Pedigree / Draw for three related Newfoundland families are shown in Appendix C.1. The simplified nuclear families are presented in Appendix C.2. and are numbered Family 1 through to Family 13 (F1 - F13).

Family 1 (F1) with individuals 2 and 3 shows mother to daughter transmission, although the status of the sire of female 3 is unknown. This is consistent with an autosomal dominant or a matrilineal mode of inheritance but does not exclude autosomal recessive or X-linked dominant modes. There were no common ancestors in the three generation pedigree for 2 although common ancestry was evident in 3. F2 and F3 had no common ancestry in five generation pedigrees. F2 (females 86 and 93) was related to the extended family under investigation, whereas F3 (female 87) was completely unrelated as far as could be ascertained. F4 (male 17) shared common ancestry. Without information in parental generations or any progeny from

F2, F3 and F4, the mode of inheritance could not be determined. F5 shows individuals 59 and 113 with confirmed DCM and dFS respectively. They are included in the discussion about F7. Sire 65, out of DCM female 59, did not fulfil M-mode criteria for echocardiographic abnormalities, but did have a large systolic LV volume. However, he also had an atrial septal defect (patent foramen ovale). The inbreeding in this family makes interpretation of actual mode of inheritance uncertain. Extension of this family, as F5b, shows that offspring with echocardiographic abnormalities (dFS) are present in other progeny of both the sire and the dam. Sire 6 is a Canadian import, illustrated further in F9. F6 (female 1) is related to the extended family by grandsire P502, although the dam was an import and no common ancestors were present. This family is not informative in determining mode of inheritance as information is solely limited to the proband.

The most informative family is F7, including DCM individuals 7, 59, 56, 70, 33, 96, 8, 116, 31, 94 & 42. In addition, colleagues had confirmed a diagnosis of DCM in three other individuals born in 1989, by P263 x 43, and P519 (P263 x 7). A number of relatives also had dFS. Most of this family is the progeny of one imported sire, P263, although this dog is related to other imported UK dogs (son of P104, a widely used stud dog). Unfortunately, no information could be obtained about this dog. On the maternal side, DCM was prevalent and was confirmed by echo and PM in dam 7, although the cause of death in this bitch was pulmonary neoplasia. Litter sister, dam 43, did not have a technically adequate scan, due to obesity, but subjectively, myocardial function was good and no abnormalities were evident at post mortem at the age of 12 years, after euthanasia for osteoarthritis. She was therefore determined to be normal. In the progeny of normal bitch 43 and P263, in two litters, there were 18 individuals (8 males and 10 females). Two males and a female were confirmed to have DCM and one dog was suspected to have DCM in the 1989 litter. One female, 59, was confirmed to have DCM in this study, and P27 had dFS. In the 1991 litter, male 56 and female 70 were confirmed as having DCM and two males (54 & 110) and two females (49 and 113) had dFS. One male had suspected DCM and a bitch died under general anaesthesia for routine ovariohysterectomy. Four individuals were

not evaluated. Affected female 7 x sire P263 resulted in 13 offspring, eight of whom (four males and four females) had confirmed DCM. Another female, euthanased due to osteosarcoma, had dFS and regional wall motion abnormalities which were shown by many of the other dogs on initial scan before repeat scan confirmed the presence of DCM. The other four progeny were not evaluated. Males and females are approximately equally affected, excluding X-linked modes of inheritance. A matrilineal mode of inheritance is excluded if dam 43 is definitely normal. If it is assumed that bitch 43 is normal, even if a carrier in the recessive model, a proportion of between 6/18 to 14/18 of her progeny being affected is not consistent with a recessive mode of inheritance, even if sire P263 was affected (d/- x d/d). Affected dam 7 x P263 resulted in 8/13 or 9/13 (69%) affected progeny, even if the individuals not screened happen to be normal. All the progeny are expected to be affected if both parents are affected (d/d x d/d). However, the ratios in this family could be achieved if the sire P263 was homozygously dominant (P263 x 43 = D/D x -/-; P263 x 7 = D/D x D/-). The ratios in the progeny of P263 mated to a normal and an affected dam imply that this dog must be genotypically affected in both recessive or dominant models.

In F8, the progeny of affected sire 56 are shown. There is one female (111) with dFS out of a dam confirmed to be normal (56 x 63), although the co-existence of mild subaortic stenosis confuses this picture. Depressed fractional shortening individuals are evident in other progeny, although few animals in litters were screened, and dams 107 and P661 were from other DCM lines also. P661 died suddenly, although was believed by the owner to have asphixiated by eating cow dung.

F9 is extended from F7. A large number of individuals were screened in this family. Progeny of affected bitch 7, affected daughter P519 and affected half sister bitch 59 all of who had progeny to imported sire 6. The progeny in this family at the time of writing were still quite young, where typically the age of onset appears to be about 8 years. If sire 6 is definitely normal, and the equivocal echocardiographic abnormalities do indeed precede the development of overt DCM, then the presence of

abnormalities in some of these progeny (between 10 out of 29 or 10 out of 43, depending if total of scans performed (29) or total number of progeny (43) are included, representing 23 - 34%) suggest an autosomal dominant mode of inheritance with complete (probably age related) penetrance. Unless the Canadian import was a carrier, despite being completely unrelated, a recessive mode of inheritance is excluded, although a number of assumptions have been made.

Families F10a, F10b, F10c and F10d show the relationship between a widely used sire, P502, who appears in a number of these pedigrees (see F1, F4 & F6 also), and sire P104, another commonly used sire who also appears in DCM pedigrees, including siring P263 (see F1, F7, F8 & F9). He is also related to P100 and P108 (see F12). Sire P292 also appears in a number of DCM pedigrees, but again was a widely used stud dog, rumoured to have died at 12 years of age due to testicular cancer. F11 shows a very complex inbred pedigree including a widely used sire, P692, who was scanned as being normal by a colleague recently, although few of his progeny have been assessed.

Another important family is illustrated in F12 and F13. Sire P1 and dam P9 had a male and female DCM case in their progeny (80 and 9 respectively); bitch 81 was assessed to be normal. P1 was from a litter where other suspected DCM cases affected another 2 out of the 7 progeny in a related mating. P9 was reported to have been euthanased due to oral neoplasia, although cardiac status was unknown. P1xP9 was also a related mating. Dog 80 has only sired two progeny, one of which was normal at two years of age. Bitch 9 had several litters, two of which were by the same (related) sire P10, and has had two cases of DCM with other siblings showing dFS or DCM. Echocardiographic abnormalities (dFS and LVE) were shown in the two out of ten individuals screened sired by an unrelated sire (P36), although this sire is descended from P502. P36 also sired a bitch with LVE (P738) which, on mating with a DCM patients produced dog 99, who, although young, had frequent ventricular premature complexes as well as dFS). One daughter of bitch 9 (97; dFS) has also been bred to a number of unrelated (imported) sires, and echocardiographic

abnormalities were evident in some of the progeny, although again, these abnormalities require to be conclusively proven to precede overt DCM.

F13 illustrates further details about this family. Bitch 81, defined to be normal, is shown in two litters. Only one dog, with mild subaortic stenosis, was screened out of 12 progeny sired by 83. However, there were two normal progeny at the age of 4 years old (75 and 84) out of 12 screened sired by (related) P25 (status unknown). Bitch 75 was mated to the imported, unrelated sire P33, and two of eleven progeny were scanned, one of whom had DCM. This "skipped generation" is not consistent with a dominant mode of inheritance, if 81 was genuinely normal, and 75 proves to remain normal throughout her life. This, however, would not be the case if P33 proves to be affected. However, it is unlikely that he will be available for scanning. Bitch 82 had left ventricular enlargement, and one DCM son (74) with the other litter-mate not screened. The sire was again P36, also present in the predecessors of DCM cases or those with echocardiographic abnormality (e.g. dam P738 in F12).

C.15. *Test for fit for a particular mode of inheritance*

The frequency of dilated cardiomyopathy in the general Newfoundland population has been estimated as 0.08 (=q).

The mean frequency in the siblings (s) was determined by averaging the frequency of the disease in the nuclear families (Table C.7.).

Table C.7.

Family	Frequency of DCM	Decimal Frequency
Family 1	1/2	0.5
Family 1	1/1	1
Family 2	2/7	0.29
Family 3	1/6	0.17
Family 4	1/6	0.17
Family 5a	1/5	0.2
Family 6	1/13	0.077
Family 7	14/18	0.78
Family 7	9/13	0.7
Family 10a	1/4	0.25
Family 10a	2/8	0.25
Family 10b	1/9	0.11
Family 12	3/7	0.43
Family 12	2/7	0.29
Family 12	4/17	0.24
Family 13	1/2	0.5
Family 13	1/11	0.09
MEAN FREQUENCY		0.356

Relative frequency = s/q = $\frac{0.356}{0.08} = 4.45$

Frequency	Frequency	Observed Frequency	Expected	Frequency	
General population	Sibs (mean)	$(^{s}/_{q})$	Dominant $(^{1}/_{2q})$	Recessive $(^{1}/_{4q})$	Multifactorial $(^{1}/_{\sqrt{q}})$
0.08	0.383	4.45	6.25	3.125	3.5

The relative frequency of this disease is below that expected for dominant mode of inheritance but above that for a recessive or multifactorial inheritance.

C.16. *Segregation Analysis*

C.16.1. *Family 7*

The results are given for F7 (Appendix C.2.).

Results of χ^2 analysis (or Fisher's Exact Test where number of observations were <5) are shown in Table C.8.

The ratio for P263 x 43 was not significantly different from the 0.5 segregation ratio for scenarios (i), (ii) or (iii), but was significantly different if all probable cases of DCM were included and unassessed individuals excluded from the data. P263 x 43 data was also not significantly different from the 0.75:0.25 ratio, except in the case of (i). The 1:0 ratio was also not significantly different from the P263 x 43 observed data when probable DCM : unassessed, or excluded individuals, but was if confirmed DCM cases versus others were considered.

The ratio for P263 x 7 was significantly different from the 0.5:0.5 ratio if confirmed or probable DCM cases, excluding unassessed individuals, were considered. There was no significant difference for these classes from the 0.75:0.25 or the 1:0 ratios.

Table C.8.
Hypothesis Testing for Mode of Inheritance for Family 7.

Test Ratio		(i)Confirmed DCM: Other				(ii)Probable DCM: Unassessed				(iii) DCM: Other, excl.)				(iv) Probable DCM: Other, excl.)			
		P263 x 43		P263 x 7		P263 x 43		P263 x 7		P263 x 43		P263 x 7		P263 x 43		P263 x 7	
		DCM	Unaffected	DCM	Unaffected	DCM	Unaffected	DCM	Unaffected	DCM	Unaffected	DCM	Unaffected	DCM	Unaffected	DCM	Unffected
	Observed	6	12	8	5	14	4	9	4	6	5	8	1	11	0	9	0
0.5:0.5	Expected	9	9	6.5	6.5	9	9	6.5	6.5	5.5	5.5	4.5	4.5	5.5	5.5	4.5	4.5
	Test		χ^2		χ^2		χ^2		χ^2		χ^2		Fishers		χ^2		Fishers
	Significance		ns		ns		ns		ns		ns		p=0.035		p=0.004		p=0.004
0.75:0.25	Expected	13.5	4.5	9.75	3.25	13.5	4.5	9.75	3.25	8.25	2.75	6.75	2.25	8.25	2.75	6.75	2.25
	Test		χ^2		χ^2		χ^2		χ^2		χ^2		χ^2		χ^2		χ^2
	Significance		p<0.001		ns		ns		ns		ns		ns		ns		ns
1	Expected	18	0	13	0	18	0	13	0	11	0	9	0	11	0	9	0
	Test		χ^2		Fishers		Fishers		Fishers		Fishers		Fishers		Fishers		Fishers
	Significance		p<0.001		p=0.039		ns		ns		p=0.035		ns		ns		ns

C.16.2. *All nuclear families*

C.16.2.1. *Only litters with at least one case of DCM were included*

There were 32 cases of confirmed DCM, and a total of 50 cases with confirmed DCM, suspected DCM or with equivocal echocardiographic abnormalities. Only seven dogs were unaffected at the time of examination, but 101 siblings were unevaluated, giving an "unaffected" total of 108 dogs. The ratio of "Affected: Unaffected" dogs (50:108) (total 158 dogs) was compared to the expected ratio of 0.5:0.5 for an autosomal dominant mode of inheritance (79:79). χ^2 analysis showed a significant difference ($p=0.001$). This ratio was also compared with the expected 0.25:0.75 for an autosomal recessive condition (39.5:118:5), which was not statistically different.

C.16.2.2. *All litters in the nuclear families were included.*

There were 32 cases of confirmed DCM, and a total of 67 cases of confirmed or suspected or dogs with equivocal abnormalities. Forty dogs were unaffected at the time of the examination and 229 dogs were not evaluated. Confirmed and probable DCM cases "affected" were compared to an "unaffected" total of normal and unevaluated dogs (ratio: 67:269; total = 336 dogs). This ratio was compared to the 0.5:0.5 ratio expected for an autosomal dominant mode of inheritance (expected: 168:168), and was found to be statistically significant different ($p<0.001$). The ratio was compared to the ratio expected for an autosomal recessive mode of inheritance of 0.25:0.75 (expected: 84:252 dogs) and no statistical difference was identified by χ^2 analyses.

C.16.3. *Results of the "Proband" Method of Segregation Analysis*

The following data used in the analysis were gleaned from the nuclear families (Table C.9).

Table C.9.

Family	Proband identity	Probands	Affected Sibs	Total Sibs
Family1	2	1	0	1
	3	1	0	0
Family 2	86	1 (2 in litter)	1	6
	93	1	1	6
Family 3	87	1	0	5
Family 4	17	1	0	5
Family 5a	114	1	0	4
Family 6	1	1	0	12
Family 7	59	1 (3 in litter)	13	17
	56	1	13	17
	70	1	13	17
	33	1 (7 in litter)	8	12
	96	1	8	12
	8	1	8	12
	116	1	8	12
	31	1	8	12
	94	1	8	12
	42	1	8	12
Family 10a	P686	1 (2 in litter)	0	3
	P704	1	1	7
Family 10b	29	1	0	8
Family 12	P1	1	2	6
	80	1 (2 in litter)	1	6
	9	1	1	6
	10	1 (2 in litter)	3	17
	36	1	3	17
Family 13	74	1	0	1
	89	1	0	10
TOTALS			108	255

$$p = \frac{\text{Affected Sibs}}{\text{Total Sibs}} = \frac{108}{255} = 0.42.$$

Presuming that a ratio of affected: unaffected sibs is 0.5:0.5 for an autosomal dominant mode of inheritance or 0.25:0.75 for an autosomal recessive mode of inheritance, and the total number of unaffected sibs is 255 - 108 = 147, the observed ratio of affected to unaffected sibs, 108:147, is compared with the expected numbers of 127.5:127:5 (under an autosomal dominant hypothesis) and 63.75:191.25 under an autosomal recessive hypothesis). χ^2 analysis showed a statistically significant difference from the autosomal dominant model ($p=0.025$) and a highly significant difference from the autosomal recessive model ($p<0.001$).

C.16.4. *Results of the Singles Method Segregation Analysis*

If only nuclear families with confirmed cases of DCM were included, there were 32 affected individuals (A), out of a total size of 137 individuals (T). There were 11 families with just one affected individual (A_1) and four families with two affected individuals (A_2).

$$\text{“p”} = \frac{A - A_1}{T - A_1}$$

$$\text{“p”} = \frac{32 - 11}{137 - 11} = \frac{21}{126} = 0.17$$

The variance (“p_{var}”) was calculated:

$$(\text{“}p_{var}\text{”}) = \frac{(T - A)}{(T - A_1)^3} \times \left\{ A - A_1 + ?A_2 \frac{(T - A)}{(T - A_1)} \right\}$$

$$(\text{“}p_{var}\text{”}) = \frac{(137 - 32)}{(137 - 11)^3} \times \left\{ 32\text{-}11 + (2 \times 4) \times \frac{(137 - 32)}{(137 - 11)} \right\}$$

$$(\text{“}p_{var}\text{”}) = \frac{105}{2000376} \times \left\{ 21 + (8 \times \frac{105}{126}) \right\}$$

$$(\text{“}p_{var}\text{”}) = 0.00005 \times 27.67 = 0.0014$$

If individuals with confirmed or suspected DCM and cases with equivocal echocardiographic abnormalities were included as affected dogs, there were 72 affected animals (A) out of a total of 285 (T), where 24 families had one affected offspring (A_1) and 12 families had two affected offspring (A_2).

$$\text{“p”} = \frac{A - A_1}{T - A_1}$$

$$\text{“p”} = \frac{72 - 24}{285 - 24} = \frac{48}{261} = 0.18$$

The variance ("p_{var}") was calculated:

$$(\text{“}p_{var}\text{”}) = \frac{(T - A)}{(T - A_1)^3} \times \left\{A - A_1 + 2A_2 \frac{(T - A)}{(T - A_1)}\right\}$$

$$(\text{“}p_{var}\text{”}) = \frac{(285 - 72)}{(285 - 24)^3} \times \left\{72 - 24 + (2 \times 12) \times \frac{(285 - 72)}{(285 - 24)}\right\}$$

$$(\text{“}p_{var}\text{”}) = \frac{213}{17779581} \times \left\{48 + (24 \times \frac{213}{261})\right\}$$

$$(\text{“}p_{var}\text{”}) = 0.00001 \times 67.584 = 0.00068$$

The null hypothesis was tested for an autosomal recessive mode of inheritance (p_0 = 0.25).

For confirmed cases of DCM:

$$Z^2 = \frac{(\text{“}p\text{”} - p_0)^2}{(\text{“}p_{var}\text{”})}$$

$$Z^2 = \frac{(0.17 - 0.25)^2}{0.0014} = \frac{0.0064}{0.0014} = 4.57$$

From χ^2 distribution tables, with one degree of freedom, this test statistic is significantly different from the null hypothesis for an autosomal recessive mode of inheritance ($0.02 > p < 0.05$).

For confirmed and suspected cases and those with equivocal echocardiographic abnormalities:

$$Z^2 = \frac{(\text{“}p\text{”} - p_0)^2}{(\text{“}p\text{”})}$$

$$Z^2 = \frac{(0.18 - 0.25)^2}{0.00068} = \frac{0.0064}{0.00068} = 7.21$$

From χ^2 distribution tables, with one degree of freedom, this test statistic is significantly different from the null hypothesis for an autosomal recessive mode of inheritance ($0.001>p<0.01$).

The null hypothesis was tested for an autosomal dominant mode of inheritance ($p_0 = 0.5$).

For confirmed DCM:

$$Z^2 = \frac{(\text{“p”} - p_0)^2}{(\text{“}p_{var}\text{”})}$$

$$Z^2 = \frac{(0.17 - 0.5)^2}{0.0014} = \frac{0.1089}{0.0014} = 77.79$$

From χ^2 distribution tables, with one degree of freedom, this test statistic is significantly different from the null hypothesis for an autosomal dominant mode of inheritance ($p<0.001$).

For confirmed and suspected cases and those with equivocal echocardiographic abnormalities:

$$Z^2 = \frac{(\text{“p”} - p_0)^2}{(\text{“}p_{var}\text{”})}$$

$$Z^2 = \frac{(0.18 - 0.5)^2}{0.00068} = \frac{0.1024}{0.00068} = 150.59$$

From χ^2 distribution tables, with one degree of freedom, this test statistic is significantly different from the null hypothesis for an autosomal dominant mode of inheritance ($p<0.001$).

C.17. *Determination of quantity, quality and structure of genomic DNA stored*

The quantity of genomic DNA obtained by calculation using the optical density at 260nm and the OD ratios OD_{260}/OD_{280} are given in Table C.10. (Volume II).

The mean ($\pm$ standard deviation) concentration of DNA in the TE was 0.18 $\pm$ 0.08 μg/μl (or 180ng/μl), with a minimum of 0.059 and a maximum concentration of 0.449 μg/μl.

The mean ($\pm$ standard deviation) ratio of optical densities at 260nm/280nm (OD_{260}/OD_{280}), as an indicator of the quality of DNA was 1.82 $\pm$ 0.57, with a minimum of 0.9 and a maximum of 5.1.

Using the EtBr 0.8% agarose gels, large structure DNA (similar to the 23130 base pair initial band of the λ *hind III* size marker) was identified, migrating only very slowly in the gel. From the band intensity, usually slightly less than that of the 23130bp band (representing 120 ng DNA), the quantity of DNA in the 1 μl placed in each lane was estimated to be between 90 and 240 ng/μl (average estimate of 100 ng/μl (data not shown). This was largely consistent with the spectrophotometric results, apart from the later samples, where spectrophotometry appeared to give greater DNA concentration than evident on the gel.

C.18. *Simulated linkage analysis on the selected group of dogs*

The dogs selected as a family subgroup were shown in Figures C.1.a., C.1.b. & C.1.c., which included the phenotype data available at the time of this analysis.

Dr. Peter Teague of the MRC Human Genetics Unit ran the simulated linkage analysis program, Simlink. If dogs with undetermined status were included as "unknown", the maximal achievable LOD score was +2.696 at θ=0.01 (i.e. marker within one centiMorgan of the disease alleles or +2.154 at θ=0.05 (within 5 centiMorgans). This is not generally accepted as being statistically significant. If

dogs with equivocal echocardiographic abnormalities were included as Affected, then a statistically significant LOD score was achievable with a marker within ten centiMorgans of the disease allele (LOD scores: +5.262 at θ=0.01, +4.447 at θ=0.05 and +2.752 at θ=0.1). A marker giving a statistically significant negative LOD score of < -2.0 had to be outwith 12 centiMorgans of the disease locus.

C.19. *Fluorescent dUTPs for labelling PCR products*

It was found that both R110 and R6G FdUTPs incorporated into PCR reactions could successfully allow detection and genotyping of the products by the ALF/ALP system, at least for the A15 microsatellite primer set and the individual five DNA samples under investigation. R110 resulted in slightly greater amplitude peaks.

C.19.1. *The value of adding T4 DNA polymerase*

For primer pair A15 and the five DNA samples assessed, visual scrutiny of the ALF gel printouts failed to identify any major difference in the samples treated after the PCR reactions with T4 DNA polymerase, although peaks not treated with T4 DNA polymerase were in many cases taller than peaks treated when comparing the same FdUTP and same DNA sample, although this was not consistent and was not judged to be significant.

C.20.1. *Taq Gold*

The experiment to assess whether the use of Amplitaq gold instead of standard *Taq* showed that for A15, A29 and A19, the products were more clear with fewer extraneous bands. F38 did not result in a detectable product with *Taq* Gold, but there was a dramatic decrease in the multibanding effect. E41 still showed heavy extraneous banding and *Taq* Gold did not result in perceptible improvement.

C.20.2. *TD PCR versus Taq Gold*

Comparison between the use of *Taq* Gold and the TD PCR technique using standard *Taq* was subjective, based on visualisation of products and any extraneous bands on a Nusieve EtBr gel by transillumination. The touchdown technique resulted in better

product detection for K200, K120 and K292 primer sets. The use of *Taq* Gold appeared to improve product definition for K212, K39, K32. Detection of products amplified using K209 appeared similar by both techniques.

C.21. *Product detection by Nusieve gel with EtBr detection*

Having used four DNA samples for each primer pair to determine whether successful amplification resulted in discernible products on the agaorse gel, the Nuseive EtBr gel was used for the primer set which had resulted in the most distinct, bright bands and obvious polymorphism on the 1.8% agarose gel. However, it was not possible to accurately determine allele size and between polymorphisms by this system.

Primers for the microsatellite PEZ18 did result in discernible polymorphisms, although this was not accurate, since sometimes three products were evident in each lane, and, without trisomy, these could not all represent the two alleles of a microsatellite, so there was some evidence of mis-priming (Figure C.3.).

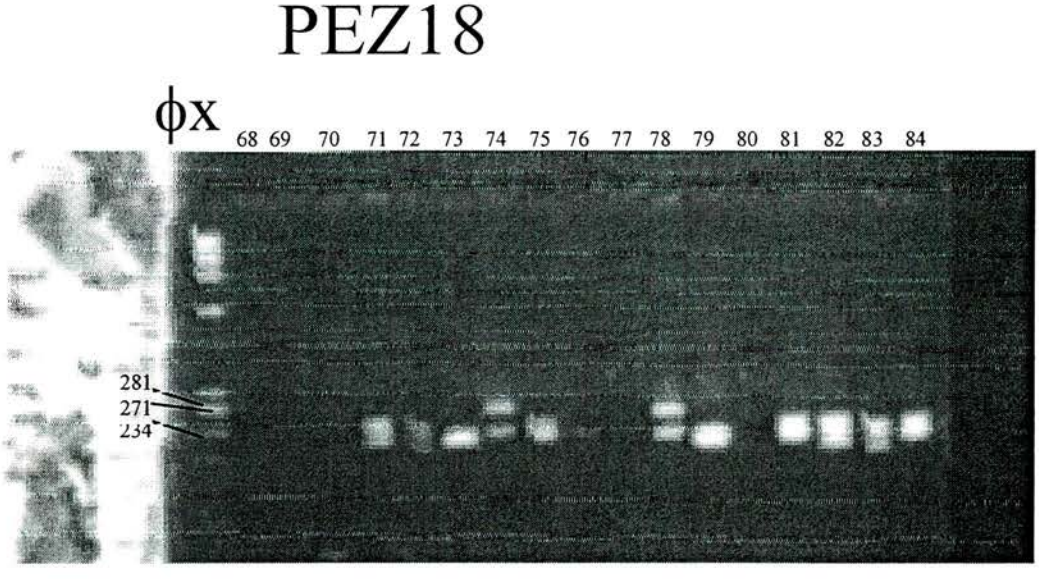


Figure C.3. Newfoundland DNA samples 68 - 84 with PEZ18.
The size marker ϕX is shown and the bands corresponding to sizes of 281/271 (close) and 234 base pairs are labelled. Although no products are evident in some lanes, there are three or more products in other lanes, indicating mis-priming.

This resulted in the need for a more sensitive technique in differentiation of alleles which only had a small size difference, hence the use of the ALF.

C.22. *Genotyping data from microsatellite primers assessed in the pilot study*

Results from analysis of PCR reaction products for each primer pair on EtBr gels and the ALF/ALP system are presented in Table C.2.b. (Volume II). An example of an ALF printout is shown in Figure C.4. (scanned image) (Volume II). Product size determined by the ALF/ALP system was similar to the log-linear derived data from the EtBr gels for microsatellites PEZ8, 2079, K298 and A15. Compared with the ALF/ALP system, EtBr slightly over-estimated product size for products of PEZ5, PEZ11, PEZ18, K336 and F17, and slightly underestimated product sizes of 2137 (although sub-family not run due to insufficient primer) and F66. The EtBr gel was unable to distinguish product due to faint product or to presence of extra bands, whereas alleles were clear by the ALF/ALP system for PEZ7 and PEZ22.

Comparison with the expected results (Table C.2.a.) reported in the literature or from personal communication from Dr. Nigel Holmes showed that similar product sizes were identified for primers PEZ5, PEZ7, PEZ8, PEZ11, PEZ22, PEZ18, 2137 (not used on entire sub-family), 2079, K336, F17 and A15. Smaller products were identified for K298 (122) as well as products similar to the reported size 148 bp product (138, 142). Smaller product sizes were also identified for F66 (167 - 175bp) than those reported (184 - 190bp).

No product could be identified for products of 2010, 2018, AHT109, AHT101, AHT110 or 2001 despite several attempts using different DNAs and different detection systems.

Products only assessed by EtBr gel showed similarity with expected product sizes for K200, K32 and A19. EtBr slightly overestimated size of products compared with reported values for K120, K207, K209, K211 and K292 and slightly underestimated

for K39. The EtBr system for 2088, E41, K212, A29, A19, E38 and F38 showed numerous extra bands making allele distinction and sizing difficult or impossible.

C.23. *Fitting genotyping data to the sub-family*

Initially, the genotype data produced by the ALF/ALP system was marked in the pedigrees of the sub-family (as in Figure C.1.a., C.1.b. & C.1.c.). If there were discrepancies identified in the family groups, the ALF print outs were scrutinised to attempt to resolve any discordant data, and genotype data were revised based on detection of presumed non-specific bands, stutter peaks, variable strand migration where both strands were labelled with FdUTPs. In general (personal communication; N. Holmes and E. Ryder, Animal Health Trust) alleles should retain similar appearance throughout a gel, and so the ALF printout appearance was also taken into account, particularly in making a decision about a homozygote or heterozygote.

The genotype data for each primer pair are detailed for each individual from the sub-family in Table C.3. The data generated from using fluorescently labelled primers are presented in Table C.3.a. and that from products labelled during the PCR reaction by FdUTPs are shown in Table C.3.b. The individuals are numbered according to the DNA number and their status correct at the time of the simulated linkage analysis and the pilot study are also shown.

Genotyping was readily achieved for PEZ11, PEZ7, PEZ8, PEZ5 and 2079. The data obtained were consistent with the pedigree data (Figures C.1.a., C.1.b. & C.1.c.) and Mendelian inheritance, as illustrated in for PEZ11 in Figures C.5.a., C.5.b. and C.5.c. (Volume II). PEZ22 also showed good fit to the pedigree, but the presence of extraneous bands meant that use of the pedigree was instrumental in some of the genotyping decisions for this microsatellite and consequently, the use of this microsatellite would prove difficult if it was used as marker to screen the general population. A15 genotype data could also be successfully fitted into the pedigree, once it was decided that low amplitude 187/191 peaks should be ignored; genuine peaks were of tall amplitude. However, only two alleles with very little

heterozygosity was evident in this family group making its use for a linkage study very limited.

Analysis of PEZ18 often showed a 215 bp peak. In the triplets (as shown in the gel illustrated in Figure C.3.), this was always the associated peak, therefore it was ignored as an extraneous band, and allele calling then depended on the residual data. However, despite considering that genotyping had been successfully achieved and checked, there were problems in matching the genotype data to the pedigree (Figure C.5. (Volume II). Individuals 32 and 45 did not fit in with parent and the inferred genotype of sire P263 based on the progeny out of dam 43 and dam 7 was inconsistent. This incompatibility is detected by the linkage analysis as an error rendering further analysis invalid.

K336 had a very complex genotype appearance. Progeny of the imported dog, 6, all showed a very complex, four peaked pattern. Because of this appearance, it was assumed that this was a heterozygote pattern, and the "double-peaked" alleles were actually homozygotes where the double strands had slightly differing migrations under electrophoresis.

It was also very difficult to interpret the ALF peaks for the products of F66. Eventually, basing genotyping largely on appearance, it could be successfully fitted to the pedigrees, with the exception of dog 45.

Initially, the ALF printouts and genotype data for K298 were also confusing. In the first instance, this was because smaller size peaks less than the selected smallest size marker were detected. The PCR products were rerun with a size marker less than these peaks. The majority of individuals showed a 138 and / or a 142 peak. In some dogs, there were peaks identified corresponding to base pair size 122 & 128, but almost all of these individuals also had a 138 or a 142 peak. Consequently, it was assumed that the first allele (arbitarily sized as the 122 peak, with an associated peak corresponding to the 128 peak which was ignored as not being an allele) was

genuine, with other potential alleles at 138 / 142. The genotype data derived from these presumptions resulted in good pedigree correlation and could be applied to other individuals.

The data from the sub-family using primers from F17 was confusing. Allele appearance was bizarre, with two major peaks, one of which was assumed to be due to non-specific priming. These were largely 152/160 or 154/162. Occasionally, a presumed heterozygote appearance was evident, with a 146 or 148 peak occasionally appearing, or a peak larger than 162, not sized by the ALP analysis (called 16X). What was apparent was that the gel appeared to change without any regard to family relationships; peaks of 152/160 suddenly in a lane changed to 154/162 for a number of lanes and then back. This shift of two base pairs appeared to be genuine on scrutiny of the ALF printouts, and, surprisingly, the points of the shifts were repeatable when all the PCR products were rerun on a new gel. For this microsatellite, no method of interpreting the genotype data would result in a fit to the pedigree.

The success or otherwise of fit of genotype data to the pedigree (i.e. consistent with Mendelian inheritance) is noted in Table C.4. (Volume II) for each microsatellite.

C.24. *Allele frequencies*

In Table C.4., the product sizes for the microsatellite primers are given, representing the potential allele sizes. The number of alleles appearing in the family was noted for each microsatellite, as well as the percentage of heterozygotes out of the 38 dogs in the selected Newfoundland sub-family. The alleles for each microsatellite was numbered from the smallest to the largest as indicated in Table C.4., and displayed for the individual dogs in Table C.5. Using the genotype data from this Newfoundland sub-family (or from all 90 dogs for PEZ11), the allele frequency in this population was determined and displayed in Table C.4.

C.25. *Linkage Analysis Data*

Data are presented in Table C.11.

Table C.11.
LOD scores calculated by MLINK for some microsatellites

	$\theta = 0$	$\theta = 0.1$	$\theta = 0.2$	$\theta = 0.3$	$\theta = 0.4$	$\theta = 0.5$
PEZ11	-999.999	-2.0793	-0.9178	-0.3466	-0.0719	0
PEZ7	-999.999	-2.389	-1.186	-0.54284	-0.1815	0
PEZ8	-999.999	-1.298	-0.521	-0.167	-0.0174	0
PEZ5	-1.269	-0.0418	0.0812	0.0703	0.0275	0
PEZ22	-999.999	-1.2387	-0.4713	-0.1697	-0.03	0
2079	-999.999	-0.1275	0.0597	0.05678	0.0205	0
A15	-0.494	-0.066	0.0238	0.0384	0.0231	0
K336	-1.0763	-0.5633	-0.273	-0.112	-0.031	0
F66	-999.999	-0.611	-0.201	-0.062	-0.0122	0
K298	-999.999	-0.775	-0.2425	-0.0473	0.018166	0

Negative graphs inferred from the data or viewed through Viewlink final.out using X-windows and the Viewlink capability offered by the HGMP Resource Centre (designed by Frank Visser) for PEZ11, PEZ7, PEZ8, PEZ22, F66 were consistent with no linkage being present. K336 was inconclusive with LOD scores between -2 and zero.

A slight positive LOD score was obtained for PEZ5 (peak LOD of +0.07 at $\theta = 0.2$), 2079 (peak LOD of 0.0597 at $\theta = 0.2$), A15 (peak LOD of +0.0384 at $\theta = 0.3$) and K298 (peak LOD score of +0.0182 at $\theta = 0.4$) which all offered inconclusive evidence for linkage. Values were very low.

LOD scores were not obtained for PEZ18 or F17 which had failed to match with the pedigrees.

PRELIMINARY WORK TO INVESTIGATE THE POTENTIAL FOR GENETIC LINKAGE ANALYSIS IN NEWFOUNDLAND FAMILIAL DCM

DISCUSSION

C.26. *Introduction*

The incidence of familial DCM in the dog is probably underestimated as the condition may exist sub-clinically in some family members and it may remain undetected unless all family members are thoroughly evaluated (Meurs *et al* 1996). As the disease appears usually in adult dogs, owners may not report back to the breeders informing them of the nature of illness or cause of death in their pet, or if they do, breeders may not collate the information as they would for a litter of puppies from each mating. Recognition of familial disease is often the first indication of a genetic causation. However, this does not exclude the possibility of a non-genetic factor common to a family of dogs which could be the primary cause of the disease (Robinson, 1991). In screening families of dogs where FDCM is suspected, it certainly will be important not to rely just on history or clinical findings, but to assess pre-clinical disease by echocardiography, as has been illustrated by human series (Keeling & McKenna 1994; Goerss *et al* 1995, Keeling *et al* 1995).

C.27. *Familial DCM in this Newfoundland population*

The disease frequency in this echocardiographic population of dogs is certainly likely to be higher than in the general population, due to ascertainment of the families. The disease was also of late onset and will result in possible underestimate of the incidence at one point in time, particularly as some dogs may succumb to other conditions and any cardiac disease is unrecognised in the occult stages.

Similar to most canine pedigrees, there was a significant amount of inbreeding evident within this population. The kinship coefficient between two parents is identical to the inbreeding coefficient of the offspring (Pedigree / Draw User's guide p. 35.). The Pedigree / Draw programme used the Quaas-Henderson iterative method

of calculating these values. This is one of several techniques used to compute inbreeding and kinship coefficients in extended pedigrees (Boyce 1983). The Pedigree / Draw software also allowed the calculation of the mean inbreeding coefficient to be recorded for each of the three Newfoundland extended families illustrated in Appendix C.1. Although for presentation purposes, these families were divided, it should be emphasised that they are all part of the same extended family. The mean coefficients of inbreeding ranged from 0.0206 (Newfoundland family 3) to 0.04364 (Newfoundland family 1).

C.28. *Pedigree Analysis*

In the results, the nuclear families were descriptively evaluated in order to infer a mode of inheritance from scrutiny of these. Matrilineal and X-linked modes of inheritance were excluded, due to apparent father to son and daughter transmission. Despite the possibility of autosomal recessive transmission mentioned in the results section, the presence of echocardiographic abnormalities which normally precede the development of DCM in progeny of completely unrelated dogs and the presence of DCM in dogs where no common ancestors were identified in the five generation pedigrees was felt to be most consistent with an autosomal dominant mode of transmission. The disease, based on the data acquired in this limited three year period, did appear to affect every generation although there was one possible instance of a skipped generation.

The limited duration of the study at the time of writing means that the equivocal echocardiographic abnormalities cannot be certain to predict DCM and future serial evaluation is still required. Sire 6, a Canadian import initially believed to be Normal, used in the major family F7, has progeny illustrated in F9. The presence of echocardiographic abnormalities in his progeny supported a dominant mode of inheritance. However, the second scan on Dog 6 showed a FS% of 19% and an increase in PEP:ET ratio, giving concern about his status. He will continue to be evaluated in the future.

There appear to be a number of instances of genetic anticipation. This may be defined as the onset of disease at an earlier age in the progeny than the parents, but may also refer to a more severe form of disease in the progeny than the parents (Mueller & Young 1995a). In F5a, female 114 is affected at a young age, and this indicates an example of genetic anticipation if dam 113 or sire 65 do progress to DCM in the future. Another possible example of this phenomenon may be shown by dog 99 in F12, the offspring of DCM dog 10, although the dam, P738 also showed equivocal abnormalities. This presumes that the ventricular arrhythmia and dFS do indeed precede the development of DCM in this individual. It may also be argued that this individual is affected at an early age, because there were abnormalities in both sire and dam and, if an autosomal dominant condition, homozygosity has resulted in earlier onset although the severity of the phenotype cannot yet be determined without monitoring his progression. The possibility of homozygously affected individuals may be present in F7, but there does not appear to be distinct ages of onset separating out in the progeny of sire P263.

In study of the nuclear families, it is also tempting to prefer the dominant mode of inheritance as most familial DCM man is transmitted as an autosomal dominant trait (Gardner *et al* 1987; Schmidt *et al* 1988; Michels *et al* 1992; Keeling & McKenna 1994; Mestroni *et al* 1994a; Durand *et al* 1995; Keeling *et al* 1995; Krajinovic *et al* 1995; Mestroni *et al* 1995; Bowles *et al* 1996; Olson & Keating 1996; Messina *et al* 1997). Cobb and colleagues (1996) reported that their investigations supported an autosomal dominant method of transmission in a family of Irish wolfhounds with DCM.

C.29. *Test for fit for a particular mode of inheritance*

Results for the test for fit of relative frequency of the disease in the affected nuclear families versus the estimated frequency of the disease in the general Newfoundland population of 8% did not confirm a particular mode of inheritance. However, the frequency was above that expected for an autosomal recessive or a multifactorial condition, and was less than that expected for an autosomal dominant condition.

These data are therefore consistent with an autosomal dominant condition with reduced penetrance. The frequency may be underestimated by the assumption that unevaluated individuals were normal. Data may be inaccurate if the estimated frequency of the disease in the population is underestimated or overestimated.

C.30. *Segregation Analysis*

C.30.1. *Family F7*

The ability to perform statistically meaningful segregation analysis was limited by the lack of families where information was available for all or most of the progeny and both parents. The late onset of the disease was also a confounding factor, as phenotyping dogs was inconclusive if they were young, and even in the case of older dogs with a normal post mortem examination, the potential for misclassification of phenotype is enormous. Family F7 was the most informative for segregation analysis, despite the lack of a confirmed phenotype or genotype in the father. All the dogs evaluated sired by P263 out of litter sisters 43 (Normal) and 7 (DCM) appeared to have confirmed DCM or echocardiographic abnormalities felt to precede the development of DCM, and other cases had been confirmed or suspected with DCM not evaluated by the author during this project. The fact that such a large proportion of the progeny were affected meant that the sire had to be inferred to be affected. It is unfortunate that no information about his cardiac status could be gleaned. In a recessive model of inheritance, two affected parents will result in all progeny being affected, unless penetrance is reduced. However, a subjectively normal although technically inadequate scan, due to obesity, and a normal post mortem in bitch 43 at an advanced age means that it is virtually certain that she was phenotypically normal. She could be a carrier in a recessive model, with 50% of her progeny being normal but also carriers. The segregation analysis was also limited, and the statistical package used noted that the Chi squared test was low power and that negative findings should be interpreted cautiously. It may be argued that unevaluated individuals should be excluded and ignored, as if they were not part of the family. This is probably reasonable in the situation of occult disease, where it is assumed

that owners will randomly decide whether or not to get the dog scanned regardless of cardiac status. There may be a bias for scanning in a population, however, if owners have slight concerns about their pet or if they are concerned about the apparent family prevalence of the disease. It may be argued that unevaluated dogs are slightly more likely to be normal than dogs submitted for scanning. However, if such dogs are excluded from the analysis and only confirmed DCM dogs are regarded as affected (situation (iii)), the Chi squared analysis is consistent with between 50 and 75% of progeny of P263 x 43 and between 75 and 100% of progeny of P263 x 7 being affected. If equivocal echocardiographic abnormalities and suspect DCM cases were included as affected (situation iv), then between 75 - 100% of progeny of both P263 matings are affected. Situation (iii) may be consistent with hypothesis 1 for the P263 x 43 mating, but not for the P263 x 7 mating. Situation (iv) however fits hypotheses 2 and 3 for both matings. In conclusion, this segregation analysis has failed to differentiate between an autosomal dominant or an autosomal recessive mode of inheritance and illustrates the difficulties in making firm conclusions from inbred dog pedigrees.

It should be considered that there may be another (non-genetic) aetiology responsible for the disease with such high proportion of the siblings being affected. However, the two bitches were in geographically different areas of the country, were fed different diets and had different lifestyles. It is hard to envisage any environmental, nutritional or viral aetiology being responsible.

C.30.2. *All nuclear families*

When Chi squared analysis was performed on all the available data from the nuclear families, the proportion of confirmed and probable DCM cases were not statistically significantly different from the ratio expected for a population with an autosomal recessive condition. However, although the bias of ascertainment was avoided as far as possible, particularly in the second method of analysis, there was potentially a huge source of error in that large numbers of unevaluated dogs were presumed to be phenotypically and genotypically normal, in favour of a large proportion of

"normals". Other sources of error include the presumption that dogs reported by the breeders to have been affected did have DCM and not another cardiac or respiratory disease, and the presumption that equivocal echocardiographic abnormalities do precede the development of DCM. These latter potential errors are in favour of increasing the proportion of affected dogs, but this is negated by the fact that the delayed age of onset and probable incomplete penetrance in this condition means that some dogs included in the analysis as echocardiographically normal at the time of examination may not remain normal. It may be argued that these results do not offer confirmation of an autosomal recessive condition. It is still possible that this ratio represents an autosomal dominant condition with incomplete penetrance, and over-estimation of unaffected individuals by including all unevaluated individuals as "normals". The results again illustrate the difficulty in confirming a mode of inheritance where knowledge of all individuals in a pedigree is incomplete. It is well understood that pooling family data is fraught because of incomplete data, possible inaccurate diagnoses and the potential for genetic heterogeneity for a specific disease (Emery 1986b).

C.30.3. *The Proband method of segregation analysis*

The p value obtained by this method, 0.42, was closer to the value of 0.5 expected under an autosomal dominant hypothesis, although the result was significantly different. However, it was very much higher than the value of 0.25 expected under an autosomal recessive hypothesis. The result obtained by this technique would be consistent with an autosomal dominant trait with reduced penetrance (known to occur, at least due to age). The result may be unreliable for two reasons. It may be overestimated by including dogs with equivocal findings or suspected cases as "affected". It may be underestimated since dogs which had not been evaluated were included as "unaffected".

C.30.4. *The Singles Method of segregation analysis*

When the Singles Method of segregation analysis was used on the nuclear families available, the major requirement for this method to be valid was that all members of

each reported family were included in the data (Nicholas 1987b). Although this was done, the family members which had not been evaluated were included as unaffected, and this results in underestimation of the proportion of affected individuals in the families. This is probably the major reason why the test results gave a significantly lower test result ("p") than the proportion of 0.25 expected under the null hypothesis for an autosomal recessive mode of inheritance and a highly significantly lower "p" result that the proportion of 0.5 required for an autosomal dominant mode of inheritance. This technique of segregation analysis was consequently uninformative. Over time in the future, it is hoped that serial evaluation and recruitment of further family members will improve the informativeness of such analyses. There are a number of more complicated methods of segregation analyses described (Emery 1986b), but the limited data available from a limited study makes these invalid at this time.

C.30.5. *The problems encountered in segregation analysis*

The method of ascertainment for families is a particularly important consideration, especially for recessive conditions. If families are only selected on the basis of one or more affected progeny, families which by chance or due to genotype do not produce affected progeny are ignored from any statistical analysis (Mueller & Young 1995b). The bias of ascertainment is much less of a problem for an autosomal dominant disease. To avoid this, all affected individuals may be assessed regardless of having any affected relatives (complete ascertainment), which is still truncate since families without affected offspring are not included in the analyses (Emery 1986b). Obviously, as not all affected Newfoundlands in the UK were evaluated during this study, the ascertainment is incomplete. Since one of the aims of the study was to generate reference echocardiographic data for this breed, and normal dogs were evaluated, some of which were not related to this extended family, it was hoped that the ascertainment of the population was not too truncated. Cases were presented individually, through the breeder, and consequently each evaluated case included here may be regarded as a proband, rather than a secondary case (i.e. one evaluated merely because of the presence of disease in a sib) (Emery 1986b). The mode of

ascertainment for this Newfoundland population may be consequently regarded as multiple incomplete ascertainment . Valid statistical analyses for such ascertainment are reported by Emery (1986b) to include the Proband method and the Singles Method (as used in this study) and the Maximum Likelihood Method, not assessed as it was mathematically too cumbersome.

These data illustrate the difficulty in statistically proving a mode of inheritance when a study is limited by (i) absence of information about all family members or all siblings in an affected family, (ii) uncertainty of phenotype in a disease with acquired onset, and initially equivocal echocardiographic abnormalities (iii) reduced penetrance (iv) the possibility of phenocopies. Particular problems in interpretation of echocardiographic abnormalities were also encountered. Elston (1992) described the use of computer software to perform a variety of more complex segregation analyses, allowing for reduced penetrance or multifactorial inheritance, although non-inbred families are preferred. Even more complex methods of segregation analyses, based on half-sibs from particular sires, mainly indicated for production characteristics of farm animals are proposed by Knott and colleagues (1991a;b).

Some of the problems encountered in genetic epidemiology are reviewed by Morton (1993). Lander and Schork (1994) discussed the difficulties in evaluating a complex trait, which was defined as any phenotype that did not exhibit classical Mendelian inheritance. Possible reasons for the absence of fit with classical models of Mendelian inheritance include the fact that different genotypes may result in the same phenotype, or the same phenotype may be due to different genotypes (as shown in human HCM and DCM), or penetrance may be low or incomplete. If there was a high frequency of disease gene within the population, even a simple trait can be difficult to map (Lander & Schork 1994), and this may be the case in this Newfoundland DCM family.

Recent evidence has also shown that a condition may behave in a non-Mendelian manner in outbred populations despite being due to a single major gene defect

(Patterson *et al* 1993). The effect of minor genes on the phenotype of conotruncal defects in Keeshonden dogs was believed to be responsible for obscuring the simple Mendelian inheritance of these defects, which were only "unmasked" after ten generations of inbreeding. It is certainly possible that acquired heart disease such as DCM may be masked in a similar manner. The authors did not speculate on the nature of these minor genes.

The various approaches to attempt to determine the mode of inheritance in Newfoundland DCM failed to confirm a particular mode of inheritance, and both autosomal dominant and autosomal recessive modes of inheritance were possible. Scrutiny of the nuclear families with disease apparent in families without any close kinship coefficient appeared to be most consistent with a dominant trait, and the test for fit for different modes of inheritance and the proband method of segregation analysis offered limited but inconclusive support for this trait.

Future work on serial evaluation of young family members and work on the progression of equivocal echocardiographic abnormalities, as well as recruitment of new family members, is planned and may help resolve some of these difficulties encountered in the simple segregation analyses attempted here.

Another major assumption made in this study is that DCM is due to a single gene defect, and is not genetically heterogeneous. As it is known that human FDCM is a genetically heterogeneous disease (Schultz *et al* 1995), this must remain a possibility. However, in a single, recently evolved and inbred dog breed, this appears unlikely.

C.31. *Determination of quantity, quality and structure of genomic DNA stored*

The mean concentration of DNA was somewhat variable. In general, there was a trend to improved concentration during the course of the study (indicated by the sequential numbers of the DNA identity code), presumably due to improving

extraction technique by the author. It was noted that samples taken early on during scanning trips, which had been stored for up to seven days prior to extraction, or samples that had been posted, had a lower yield. However, the EtBr agarose gel results suggested that these later samples were not genuinely of significantly higher concentration. Spectrophotometry tends to overestimate concentration of DNA and this is particularly so as the machine becomes hot, and this may be responsible in part to the higher DNA concentrations shown for the later samples.

Although the mean ratio between the optical densities at 260nm/280nm was consistent with pure DNA, at 1.82, there was considerable variation in this figure. Some values were significantly lower than this, indicating protein contamination, and this was felt to be a problem due to inexperience particularly with the earlier samples. However, there were occasional samples with very high ratio. This was felt to be due to contamination by the Nucleon silicon resin, which may have been retained in some samples, although this will not interfere with any molecular biological procedure, according to the manufacturer's details.

C.32. *The simulated linkage analysis data*

This subgroup of dogs was selected prior to detailed echocardiographic analysis, based on the initial assessment of the scans. The final status of the dogs was not determined, and in some cases, the final phenotype of the dogs may not be determined for some years. Because there are a number of common ancestors, the assumption of an autosomal dominant mode of transmission may not be correct. It is also unlikely that the penetrance in this condition is actually complete, particularly for younger dogs. This family was also not optimal because there were no equivalents of nuclear families with phenotype and genotype data from both parents. Consequently, there were a large number of factors confounding linkage analysis, especially simulated linkage analysis, in this group of dogs. They were, however, believed to be the most informative at the time of this pilot study. Although some significant LOD scores were obtained by the simulated analysis, these were at very small values of theta; i.e. a microsatellite would only be linked with the disease in

this small family if very close to the disease gene (within ten centiMorgans). When it is considered that the resolution of the canine genome map consists of markers a mean distance of 14.03cM with thirty linkage groups and eleven unlinked markers (Mellersh *et al* 1997), it can be seen that identification of a linked marker with this data would be fortuitous indeed. Since this initial simulated linkage analysis, detailed echocardiographic analysis has been carried out and a number of dogs have had repeat scans allowing the pedigree data to be updated. In addition, new family members have been recruited, and if this analysis was repeated, it is likely to be more informative. Parallel advances in the canine genome map as well as FDCM in man mean that prospects are continually improving for this study.

C.33. *Optimisation of PCR conditions*

Although the PCR conditions advised by the Animal Health Trust were used in this study, in fact conditions rarely are completely reproducible in different laboratories or with different thermal cyclers. Although some primers were assessed using different *Taq* (Amplitaq and Amplitaq Gold) and whether Touchdown PCR was an advantage, no attempt to optimise PCR conditions by adjusting annealing temperatures or magnesium concentrations was made. Undoubtedly, this would be worth doing. Some methods used in optimisation of PCR reactions are detailed by Kidd and Ruano (1995). The use of Touchdown PCR may assist in preventing mis-priming even if the correct discriminatory annealing temperature had been determined by avoiding secondary problems such as inconsistency of well temperatures in the PCR block or between thermal cycling machines (Don *et al* 1991). At the time of this pilot study, we were unaware that the Animal Health Trust routinely used Amplitaq Gold. However, for the primer pairs requiring standard PCR showing extraneous banding with standard *Taq*, improvement was identified in most of these, with greatly reduced banding. Another comparison was only between touchdown PCR with normal *Taq* or standard PCR with the reported annealing temperature with *Taq* Gold. It was not assessed whether product definition or detection would be improved by the use of both techniques together (*Taq* Gold with TD PCR). It is probable that this will improve the results and consequently, future

work with difficult primer sets known to produce extraneous bands will use both *Taq* Gold and the TD PCR technique.

To prevent mis-priming events, adding the *Taq* polymerase only after the reactions had initially been heated to 94°C was another solution (Thomas *et al* 1997) (called Hot Start PCR), although this is made unnecessary if *Taq* Gold was used.

C.34. *Fluorescent dUTPs for labelling PCR products*

The experiment to assess the ALF/ALP system's ability to detect and genotype PCR products incorporating FdUTPs was successful for both R110 and R6G. As R110 has slightly greater amplitude peaks and it was cheaper, this was then selected for the unlabelled primers to enable the ALF/ALP system rather than other techniques such as autoradiography to be used to genotype products. Schwengel and coworkers (1994) showed that both techniques were similarly successful. Although incorporating FdUTPs increased the cost of the PCR reactions, ordering fluorescent 5'end-labelled primers would be significantly more expensive than this. However, primers which have been labelled during synthesis have only one strand (the forward primer) labelled, so that only one strand of the microsatellite product is labelled. Incorporation of FdUTPs in the PCR reaction results in labelling of both oligonucleotide strands, which may have slightly variable mobility, so that peaks may appear broad and it may be difficult to differentiate allele sizes different in size by less than four base pairs (Rhodes *et al* 1997). This was certainly evident with many of the primers assessed in this pilot study, particularly those amplifying dinucleotide repeats. This variable mobility may in part be due to the differing content of nucleotides of complimentary strands. For example, in a dinucleotide repeat microsatellite $(CA)_n$ with complimentary $(TG)_n$ strand, FdUTP is preferentially incorporated into the $(TG)_n$ strand as U is complimentary to A. $(CA)_n$ strands move more quickly than $(TG)_n$ strands (Litt 1991) and it can be appreciated that FdUTP will result in a stronger signal due to greater labelling of the $(TG)_n$ strand.

High throughput genotyping of microsatellites for linkage to human disease has been made possible by incorporation of sets of chromosome specific marker loci and use of multiplexing PCR or multipooling of products onto a single gel (Reed *et al* 1994). This requires reliable and uniform PCR reactions and it appears unlikely that such an approach is suitable for a canine linkage study, due to the cumbersome PCR conditions required by some primers for canine microsatellites and the difficulty in accurate genotyping of many of these microsatellites. The canine genome map is also not currently sufficiently defined at the stage to allow such an approach, and it was not assessed during this pilot study.

C.34.1. *The use of T4 DNA Polymerase*

The conclusion was made that the addition of T4 DNA Polymerase to PCR products labelled with FdUTPs did not have a significant effect on the alleles detected and genotyped and the ALF/ALP system for the primer pair A15 and the five DNA samples assessed. This suggested that *Taq* polymerase addition of one base to amplified products was not a major confounding feature to allele detection or genotyping for this primer pair (Ginot *et al* 1996). However, A15 resulted in peaks that were less broad and less equivocal than some of the other primers which were supplied unlabelled, and it may, in retrospect, have been useful to assess the importance of single base addition and T4 DNA polymerase on some of the more difficult primer sets.

The problem with this addition of single bases to the 3' end of PCR amplified products is that not all strands receive the extra base (which is usually adenosine). In part, the final ten minute 72°C extension phase of the PCR conditions is to allow many of the strands to achieve this extra base addition (Magnuson *et al* 1996), but for some primer sets, there is still a considerable mix of products, particularly depending on the terminal 5' sequence of the reverse primer (Smith *et al* 1995; Magnuson *et al* 1996). An alternative solution to this problem is to provide reverse primers with a tail 5'sequence such as GTTTCTT which results in adenylation of the majority of the strands (Brownstein *et al* 1996). Such modification means that the complex

electrophoretic pattern is minimised. This manipulation of the primers is called pigtailing (Brownstein *et al* 1996).

C.34.2. *Other artefacts encountered during genotyping with FdUTPs*

Other artefacts of *Taq* polymerase which complicate the migration profile of microsatellites, particularly the dinucleotide repeats with only small difference in allele sizes, include stutter. Stutter peaks are usually small peaks preceding each actual allele, which are multiples of two base pairs smaller than the actual allele, and a number of stutter peaks may be evident preceding each allele. Stutter peaks mean that the smaller allele is often of greater amplitude than the larger allele in a heterozygote due to the concommitant stutter peak of the larger allele (Genotyper v2.0 Handbook, Perkin Elmer). This phenomenon is due to slippage of *Taq* polymerase during PCR (Ginot *et al* 1996). In general, an awareness of the problem allows accurate genotyping despite the stutter peaks, and in semi-automated genotyping like the ALF/ALP system, stutter peaks are ignored by the software by setting the amplitude of stutter peaks to be ignored (usually 70%). The use of FdUTPs incorporated in the PCR reaction does, however, does make differentiation between stutter peaks, heterozygote alleles with only small size difference and addition of single bases resulting later peaks much more difficult, since both DNA strands are labelled and may have slightly non-uniform migration. In some cases, a large broad peak which was virtually impossible to genotype resulted.

The extra peaks or bands due to *Taq* slippage are reported to be more common at higher temperatures (Litt 1991), and it can be readily appreciated that the high annealing temperatures or touchdown PCR techniques required by some these primer sets may exacerbate this problem. In contrast, spurious priming resulting in extra bands is more likely to occur at lower temperatures (Kidd & Ruano 1995).

C.35. *Genotyping data for microsatellites*

The log-linear plot using the size standard ladder ϕX174 *Hae*III and measuring migration distance proved to be fairly successful in estimating the expected product

size, although it tended to overestimate product size compared with reported data or that obtained by the ALF/ALP system. However, it was certainly useful as a guide prior to running the sub-family on the ALF/ALP system, in that internal size markers for each lane (smaller than and larger than expected product size) had to be selected and loaded. The EtBr detection system and Nuseive gel did not, however, prove to be sensitive enough to distinguish between polymorphisms or even to identify heterozygotes versus homozygotes.

It proved to be very difficult indeed to genotype the products of some primer pairs, particularly if they were labelled during the PCR reaction by FdUTPs. Even discussion with Dr. Nigel Holmes and Ed Ryder, very experienced in the interpretation of FdUTP labelled products, did not always confirm a method of genotyping that would fit to the pedigree, possibly since they were unused to the ALF/ALP system but were very familiar with the more widely used ABI system. Although a considerable amount of time and effort went in to genotyping the primers used for the individuals in the pilot study, this may not be a solution for a full linkage study. If a microsatellite is difficult to genotype, then even if it is significantly linked to the affectation status, this marker would not prove to be useful as a screening tool for the population at large unless the majority of individuals can be unequivocally genotyped. In species where there are a large number of microsatellites identified and comprehensive linkage maps published such as the mouse and human, primers with demanding conditions or equivocal genotype data are discarded.

The microsatellites and the appropriate primer sequences published by the Animal Health Trust (Holmes *et al* 1993; 1994;1995 ; Thomas *et al* 1997 were obtained by screening clones from a canine genomic library in pWE15 (Stratagene) or M13mp10 and sequencing the dinucleotide repeat sequence and flanking regions for the generation of appropriate primer sequences. Consequently, clones are available for each microsatellite. One solution for the difficulty in genotyping with FdUTPs is to run the clones on an ALF and assessing the resulting appearance and genotype data

with the known characteristics of each clone; i.e. whether homozygous, heterozygous etc.

It is possible to recognise mutation events in microsatellites, when they are followed through the generations (Brinkman *et al* 1998). In the example illustrated in Figure C.6., for the pedigree data for PEZ18 with the sire P263, one may argue that the novel allele 127 detected in individual 54 may be a mutation. If this dog had genotype 231/235, then he would be consistent with the other progeny and the inferred genotype of the sire P263 would be consistent with his progeny out of the other dam. However, when considering this particular microsatellite, genotying was problematical and there were other discordant individuals also (dogs 32 and 45) making it more likely that the incompatibilities identified were due to genotyping error rather than inherent mutation events.

It was interesting to note that the sire imported from Canada (individual 6) often had very different microsatellites from the major part of the family. In general, this resulted in his progeny being very informative, although most of them are not currently old enough to finally determine their phenotype with regard to DCM.

C.35.1. *Allele frequencies and heterozygosity*

The problematic dinucleotide repeat amplified using F17 (=AHT133) was reported to have five potential alleles and expected product size of 154 - 162, with a PIC value of 0.6 (Holmes *et al* 1995). Consequently, it was very disappointing that in this family it proved to be uninformative. A15 (reported as AHT104) had four alleles and product size of 173 - 191 reported by Holmes and colleagues (1995) with a less than optimal PIC of 0.45, so that it was not surprising that in this Newfoundland subfamily, very little heterozygosity was identified. The other primer pairs assessed on the ALF had not been reported with their number of alleles and their PIC, so comparison was not possible. However, it is only to be expected that a relatively uncommon breed with presumed small gene pool with a high degree of inbreeding does show a high degree

of homozygosity; it was surprising that the genotyping data for some of the microsatellites at least showed a reasonable percentage of heterozygous individuals.

C.35.2. *Microsatellites*

For a microsatellite to be informative in a linkage analysis study, a high degree of polymorphism is required. This can be determined by an analysis of allele frequency in a panel of unrelated mixed and pure bred dogs and it is expressed as the PIC value (Francisco *et al* 1996) which is published with the primer conditions for all the published microsatellites (see Table C.2.a.). The PIC content is calculated from the proportion of individuals homozygous and heterozygous for each allele of known frequency using a formula and computation detailed by Strachan and Read (1996d). The PIC varies between zero and one, where PIC = 1 represents a perfectly informative marker. To be useful, markers should have PIC>0.5 with PIC>0.7 being optimal (Ostrander 1998).

Dinucleotide repeat sequences are much less variable in pure bred dogs than in mixed breed dogs and this may make them unsuitable for a linkage analysis study (Mellersh *et al* 1997). However, the tetranucleotide repeats are generally more polymorphic, as shown by their PIC value in Table C.2.a., although they do have higher mutation rate than the dinucleotide repeats (Francisco *et al* 1996) resulting in detection of non-parental alleles in some progeny. This mutation rate may differ for different alleles at the same locus and is more likely to occur in the male germ line (Brinkman *et al* 1998). There was no conclusive evidence of mutation identified in this study.

C.36. *Linkage analysis and LOD scores*

No significant LOD scores were obtained for the ten primers assessed with informative genotyping data. The majority of the microsatellites were conclusively not linked to the disease, assessed by maximally negative LOD scores at zero theta. Some microsatellites were associated with inconclusive LOD scores which even became slightly positive. However, these were PEZ5, 2079 and A15 and K298, which had major allele only (PEZ5 and 2079 and K298) or minimal heterozygosity

(A15) which meant that the equivocal LOD scores were a function of the uninformativeness of the markers rather than the family.

It would have been exceptionally fortuitous to identify significant linkage in a pilot study where only ten markers could successfully be used for two-point linkage analysis. In the linkage studies described for DCM in humans, a significant proportion of the genome was screened, from 30% (Kass *et al* 1994) up to 95% (Krajinovic *et al* 1995).

In the inbred pedigree present in this Newfoundland family, with common ancestors and high degree of consanguinity for some matings, there must remain some uncertainty about the proposed mode of inheritance in this study (presumed to be autosomal dominant). An autosomal dominant mode of inheritance is by far the most common reported in the human literature for non-syndromic DCM. However, in human families, it is probable that any autosomal recessive transmitted disease is regarded as sporadic since single cases in a family will not be regarded as a familial disease and ascertainment bias confounds recognition of this transmission (Strachan & Read 1996e).

It is rarely possible to be completely certain about a mode of inheritance of a character simply by inspecting a pedigree, particularly if many of the progeny are not screened (or in small human families). In mice, test crosses would be carried out to check for ratios of affected: unaffected to confirm a particular mode of inheritance (Strachan & Read 1996e).

The use of two-point and multipoint linkage analysis is dependent on correct assumptions of modes of inheritance, penetrance and genotype frequencies in the population. In an acquired disease with delayed and variable age of onset and slow progression from initial equivocal echocardiographic abnormalities, and in a species where genotype frequencies for microsatellites are not widely published, let alone in a specific breed, inevitably a linkage analysis study may be flawed. One technique

which may be useful is affected sib pair analysis, which does not require any assumptions and is a powerful tool in the investigation of complex disease (Kruglyak & Lander 1995). Software for this analysis (MAPMAKER/SIBS) is available at HGMP-RC. Another new non-parametric analysis has been proposed for complex traits supported by the GENEHUNTER computer program which is less sensitive to mis-specification of the linkage model (Kruglyak *et al* 1996). This has an advantage over sib-pair analysis in that other pedigree information is utilised. This is available at HGMP-RC and future work using this program may be very useful. However, an alternative approach suggested by Greenberg and others (1998) was to use simple LOD score analysis and a limited set of simple genetic models even for complex diseases.

C.37. *The future and the potential for further investigation of Newfoundland familial DCM*

Identification of a microsatellite marker linked to DCM in this family will allow screening for the disease prior to the development of any echocardiographic abnormality or other evidence of the disease being present. How accurate this is in correctly identifying individuals who do go on and develop the disease will depend in part on how tight the linkage is. If the marker is sufficiently distant from the putative DCM disease gene, recombination may occur between the two allowing some individuals who never develop the disease to be incorrectly labelled, or some "clear" individuals may go on and develop the disease, having been used for breeding. Recombination has been a problem in the use of a microsatellite marker for copper toxicosis in Bedlington terriers (Yuzbasiyan-Gurkan *et al* 1997; Holmes *et al* 1998), although it is still regarded as a useful diagnostic test, particularly in comparison to the invasive alternative of liver biopsy.

At this stage, identification of a microsatellite by two-point linkage analysis may not result in identification of the gene defect responsible for DCM in this Newfoundland family. The canine genome linkage map is not densely packed with markers and multipoint linkage analysis therefore may not be applicable. It is only once a

relatively small distance of the genome has been localised by multipoint linkage analysis that a positional cloning technique may be used in order to identify a known or a novel gene associated with the disease. Few actual genes have been mapped onto the canine genome map (Mellersh *et al* 1997), and it is probable that it will be by investigation of synteny between genomes of the dog and other, well mapped species such as the mouse and human that gene loci will be identified. So far, human chromosome 17 loci have been reported to be syntenic with regions of dog chromosomes 9 and 5 (Werner *et al* 1997). Other parallel advances required for future work in the genetics of canine DCM include karyotyping of the canine chromosomes, an area currently under active investigation (Fischer *et al* 1996; Langford *et al* 1996; Thomas *et al* 1997).

A canine BAC library has been recently imported into the UK and is a resource held by HGMP-RC. Bacterial artificial chromosome (BAC) vectors are used for DNA cloning and in this case it has been applied to canine genomic DNA from a male Dobermann, cloned in the pBACe3.6 vector. This may be of concern in investigating DCM using this particularly canine BAC library because of the prevalence of DCM in this breed.

C.38. *General discussion about the genetics of DCM*

There are a number of possible candidate genes that may be associated with DCM. Genetic evidence available to date implicates abnormalities of genes encoding cytoskeletal proteins in naturally occurring DCM as well as experimental models. These include dystrophin abnormalities (Stevenson *et al* 1998), actin (Olson *et al* 1998), abnormalities of the dystrophin associated glycoprotein complex, such as sarcoglycans (van der Kooi *et al* 1998), adhelin (α sarcoglycan) (Fadic *et al* 1996), probably tafazzins (D'Adamo *et al* 1997) and possibly emerin (Cartegni *et al* 1997). In a single DCM patient (of 23 screened), deficiency of the cardiac specific cytoskeletal protein, metavinculin, was identified (Maeda *et al* 1997). Metavinculin is an isoform of vinculin, and is located at the intercalated disc and the subsarcolemmal attachment sites of non-sarcomeric actin.

Further support of the importance of the cytoskeleton in DCM was gained when the gene involved in autosomal recessive cardiomyopathy in the Syrian hamster, was identified to be a fairly cardiac specific δ-sarcoglycan gene mutation, resulting in absence of the element from the myocardium and depletion in the skeletal muscle (Nigro *et al* 1997).

The dystrophin associated glycoprotein (DAG) complex spans the sarcolemma to provide linkage between the subsarcolemmal cytoskeleton and laminin, a major constituent of the extracellular matrix (ECM) (Fadic *et al* 1996). Several different muscular dystrophies are the result of disruption of different components of the link between the muscle cytoskeleton and the extracellular matrix. Fadic and colleagues (1996) postulated that the structurally weakened sarcolemma may rupture under mechanical stress, allowing calcium and other extracellular components to enter the cell, which may eventually result in cell death. It seems reasonable to presume that a similar mechanism may also result in DCM if the primary defect was in one of the cytoskeletal proteins.

Rampazzo and others (1994) also noted that the dystrophinopathies are possibly related to a stretch modulated Ca channel. The right ventricle is particularly sensitive to stretch, as putatively manifested in the early involvement of the RV in Becker's muscular dystrophy, and these authors thought that the ARVD gene may be an as yet unknown component of the cytoskeleton specific to myocardial cells. In a later publication identifying a fourth ARVD locus, Rampazzo and colleagues (1997). observed that family members with active sport involvement had a more severe manifestation of symptoms than those more sedentary family members. It is known that exercise results in volume and pressure overload of the heart.

Mutations in the muscle LIM protein, encoded by the *MLP* gene, have been shown to result in DCM in mice (Arber *et al* 1997). Muscle LIM protein is thought to serve as a scaffold for organising the assembly of contractile proteins into sarcomeres along

the actin cytoskeleton (Leiden 1997). MLP -/- mice show similar perturbed cytoarchitecture as human DCM, although histological findings in the mouse model are more dramatic. MLP immunoreactivity shows the protein to be associated with the Z lines of the myofibrils, associated with vinculin (known to be involved in anchorage of the actin based cytoskeleton to the cell membrane) (Arber *et al* 1997). Z line associated structures are responsible for the lateral alignment of myofibres and their lateral anchorage at N-cadherin and vinculin containing costameres along the cell membrane. MLP deficient mice show disruption of the Z lines, and the gap junctions, identified by connexin 43 immunofluoresence, are less well ordered (Arber *et al* 1997). Similar changes in the cardiomyocyte ultrastructure was reported in naturally occurring human DCM when comparing explanted human hearts with normal pig hearts (Schaper *et al* 1991). Arber and others (1997) postulated that increased load after birth resulted in systemically acting factors and stress sensors in cardiomyocytes which induced and controlled a hypertrophic response. In the absence of MLP in this mouse model, the deficits in cytoskeleton architectural organisation resulted in impaired cell and tissue tension, so that, in the presence of hypertrophic stimuli, the cardiomyocytes expanded but failed to generate sufficient tension to control the hypertrophic response eventually resulting in hypertrophy (increased heart mass) and dilatation consistent with DCM.

These cytoskeletal proteins act as a scaffold for force generation by interacting with myosin (actin) or transmitting force to the extracellular matrix or adjacent cardiomyocytes. Despite initial logical suggestions (Mestroni *et al* 1994), the defect with DCM does not generally appear to be an abnormality of force generation or sarcomeric proteins themselves. The hypothesis postulated by Olson and colleagues (1998), when discussing actin mutations, that DCM results from an episodic defect in force transmission, predisposing cardiomyocytes to mechanical injury and cumulative cell death, secondary interstitial fibrosis and cardiac dilatation, would appear to be logical to the other cytoskeletal abnormalities, whether naturally occurring or experimental. Exertion, by resulting in volume or pressure overload on

the cardiomyocytes with altered function, may accelerate this degenerative process (Stevenson *et al* 1998).

Kaprielian and colleagues (1998) showed that DCM resulted in increased cell length compared with ischaemic cardiomyopathy, where cell slippage was the mechanism for cardiac dilatation. In DCM, maintenance of myocyte spatial relations with increased cell length may be explained by cytoskeletal protein abnormalities, and may explain the apparent cyclicity of echocardiographic changes in serial evaluation of relatives with abnormalities possibly preceding occult DCM. Cardiomyocyte slippage in ischaemic myocardial disease precludes the possibility of recovery of function.

Unravelling the genetic defect(s) behind the cytoskeletal abnormalities associated with DCM may result in better understanding of cardiomyocyte structure and function, which could potentially direct novel treatments directed against the pathogenetic mechanism resulting in cardiomyocyte damage. The Syrian hamster model of cardiomyopathy is associated with absolute deficiency of δ-sarcoglycan and depleted sarcoglycans and α-dystroglycan. It has been shown that adenoviral construct containing human δ-sarcoglycan can effectively restore all the sarcoglycans and α-dystroglycan, showing the potential for in vivo gene therapy (Holt *et al* 1997).

The extracellular matrix (ECM) proteins are also believed to be important in DCM. Collagen sub-types I and III are essential components in the ECM. Characteristic differences have been reported in the ECM in a variety of heart diseases including DCM. In DCM, the ratio of collagen subtypes I:III is increased, which is not correlated to the degree systolic dysfunction (Pauschinger *et al* 1998) but it may be associated with diastolic dysfunction or decreased LV compliance (Marijianowski *et al* 1995). Collagen is believed to be the major structural component determining the architecture and functional integrity of the myocardium (Gilbert & Wotton 1998). Collagen struts and tethers interconnect myocytes and prevent cell slippage. The presence of collagenolytic enzymes, promatrix metalloproteinase-9 activity and

neutrophil elastase actvity, suggests that increased levels in Dobermann DCM may reflect structural remodelling (Gilbert *et al* 1997). The elevation of these enzymes also supports the presence of inflammation (despite no histological evidence of an inflammatory infiltrate) (Gilbert *et al* 1997) which Baig and colleagues (1998) speculated may be the cause of left ventricular enlargement early in the course of human occult DCM.

The cause of apparent upregulation of the genes encoding ECM proteins may be the result of other substances, particular transforming growth factor β_1 ($TGF\beta_1$), angiotensin II, aldosterone, noradrenaline and endothelin. What is unclear is whether the changes in ECM proteins cause the heart failure or whether they are the result of some other primary process. It is apparent that neurohormonal factors are important in the interaction between the cardiomyocyte and ECM (Boluyt & Bing 1995).

The observation by Tidholm (1998) and Tidholm and colleagues (1997; 1998a;b) that histological evidence of attenuated wavy fibres is a diagnostic criterion for the presence of DCM with high sensitivity and specificity is controversial and is dismissed as fixation artefact by some pathologists (R. Else, personal communication). However, it is conceivable that this finding is the result of altered cytoskeletal or ECM function and cardiomyocyte slippage.

SECTION D

CONCLUSION

The limited evidence from this study suggests that the echocardiographic diagnosis of left ventricular enlargement or depressed fractional shortening do predict the more conventionally accepted findings of occult DCM. However, this is based on limited serial evaluation over a relatively short time course (maximum three years). Future evaluation of dogs in this study will confirm or refute these findings. A longer term study is certainly indicated, in that the progression of early echocardiographic abnormalities to DCM appears to be slow in this breed, which normally has relatively benign disease, compared with other breeds such as the Dobermann.

Serial echocardiographic evaluation is also indicated in this family in order to conclusively determine a phenotype for most individuals, so that the future work with two-point genetic linkage analysis is robust. It is necessary to be certain about the mode of inheritance and the true prevalence of the disease to enhance the prospects for identifying a marker, significantly linked to the disease, which may be used for future screening of this population.

Early identification of occult DCM or predictors of this condition is important. There are general management reasons for this. The owners knowledge will result in better observation and improved recognition of early abnormalities suggesting that the dog is decompensating. The dog's veterinary surgeon was routinely informed about abnormalities once the diagnosis of occult DCM was not equivocal. This resulted in improved patient monitoring and special care taken, for example if the dog required a general anaesthetic. The recognition of occult disease also has a bearing on the timing of therapeutic intervention.

The results of this study are consistent with the conclusions of McMurray and others (1998), that screening for asymptomatic left ventricular dysfunction to prevent heart

failure is worthwhile, although it is arguable that echocardiographic screening is cost effective in both canine and human cardiology.

There has been some evidence that Newfoundland with DCM in the USA may be taurine deficient and may respond to taurine and L-carnitine supplementation (Sisson, D., Veterinary Cardiology Email discussions). Newfoundlands may have cysteinuria (Casal *et al* 1995), which may result in urinary loss of taurine or L-carnitine, with consequences on myocardial function. This has been reported in a Dachshund (cited by Kittleson 1998). One Newfoundland was reported in a group of other breeds with cysteine or urate urolithiasis, where 13/15 dogs were identified to be taurine deficient, although it was not clear whether the Newfoundland was affected (Pion *et al* 1998). Seven of these dogs had or developed DCM, although again it was unclear whether this included the Newfoundland. In the future, it will be important to assess plasma taurine levels in cardiomyopathic Newfoundlands and this is proposed.

The new index of combined systolic and diastolic myocardial performance proposed by Tei and colleagues (1995) appears to be a promising method of distinguishing between normal and abnormal myocardial function. There have been no reports of its use in patients with very early DCM. Future studies will evaluate this index in normal and conclusively cardiomyopathic Newfoundlands and Newfoundlands in the other categories.

It is slightly concerning that two manifestations of DCM were recognised in Newfoundlands. One form was associated with marked left ventricular dilatation and hypokinesis, with proportionate left atrial enlargement. The other was associated with minimal left ventricular dilatation, less severe hypokinesis but marked left atrial or biatrial enlargement. Although both forms occurred in the same family, for future genetic analysis it is important to validate that these are both different manifestations of the same disease, presumed to be due to the same gene defect. If this is not the case, then progress in genetic linkage analysis will be slowed. The description of

mildly dilated congestive cardiomyopathy (MDCM) in man suggests that these cases, which may be similar to the Newfoundlands with minimal left ventricular enlargement, often do have a positive family history for classical DCM (Keren *et al* 1990) and it may be that this is variable manifestation of the same disease. There was minimal or no myofibrillar loss in the MDCM patients, which finding the authors believed may have been responsible for the preservation of fairly normal cardiac size in this group, despite deteriorating haemodynamic status and eventual death due to intractable congestive failure.

In most Newfoundlands in this study, DCM was a late onset disease. This resulted in difficulty in younger dogs in determination of the phenotype. Serial evaluation of such individuals through their lives will aid this final phenotyping. In a study over a limited time period, such as the three year study reported here, it is not possible to confirm with certainty that the dogs with equivocal echocardiographic abnormalities such as depressed fractional shortening or left ventricular enlargement do indeed all progress to DCM. Some of these findings may be limited manifestations of the disease, as suggested in human relatives of DCM patients (Baig *et al* 1998). There may be a continuous spectrum of phenotype from the grossly normal heart to the overtly cardiomyopathic, as described in genetically determined human hypertrophic cardiomyopathy (Hagège *et al* 1998). New, more sensitive diagnostic criteria may be indicated. Although this had been an initial aim of this study, because of the slow progression of equivocal echocardiographic abnormalities to overt DCM in Newfoundlands, this was not possible over a three year time period, but it is hoped that such guidelines can be provided for this breed with future evaluation of these individuals.

This study has laid the foundation for a genome-wide linkage analysis study and other molecular genetic techniques in the investigation of FDCM in the Newfoundland breed, which may have implications for improved understanding of the aetiopathogenesis of this condition, its diagnosis and treatment.

In conclusion, this study has confirmed that DCM is a familial disease in the Newfoundland breed, with probable autosomal dominant mode of inheritance. Echocardiographic abnormalities do precede the development of DCM, but at this stage, it is not possible to state that they are pathognomic for incipient disease. Future work is indicated and will be carried out with serial evaluation of family members showing equivocal echocardiographic abnormalities in order to confirm phenotype and determine the progression of DCM through the earliest stages. New family members will be recruited in order to attempt to define the mode of inheritance more conclusively. The family is suitable for a linkage analysis study.

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ABSTRACTS

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